The Isolation and Characterisation of Pathogenic Clostridia from Human, Equine, Avian and Environmental Sources.

Ann Heffron

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2008.

Contents

5.1.3 Detection of the C2 toxin genes and novyi alpha toxin gene by PCR 132

Abstract

Equine grass sickness is a fatal dysautonomia. The aetiology of the disease is unknown. It is characterised by severe neuronal degeneration and neuronal loss in both the enteric and autonomic nervous systems. The current hypothesis is that equine grass sickness is caused by a toxicoinfection with *C. botulinum* type C. This study has investigated the isolation and characterisation of organisms resembling *C. botulinum* type C from the gastrointestinal tract of horses with grass sickness and of control horses and the detection of the botulinum type C neurotoxin (BoNT/C) in the GI tract of horses by molecular methods.

No organisms typical of the Group III clostridia were isolated from the GI tracts of horses though organisms were isolated that were atypical with toxin gene profiles that did not appear to match their phenotype. Five of the organisms isolated from the GI tract of horses with equine grass sickness were found to produce detectable levels of BoNT/C by ELISA. The BoNT/C gene was detected by PCR and Southern blotting in four of the seven horses with equine grass sickness and none of the control horses investigated. The BoNT/C gene was detected in several of the sites sampled along the GI tract and was detected in the duodenum of all four horses. These results support the hypothesis that a toxicoinfection with *C. botulinum* type C is associated with equine grass sickness.

C. botulinum type C is one of the Group III clostridia comprising *C. botulinum* types C and D and *C. novyi* type A. These organisms are phenotypically similar and can only be identified to species level on the basis of the major toxin they produce. The

genes for these toxins are carried on pseudolysogenic phages and are readily lost. The chromosomally encoded C2 toxin is the other major toxin produced by this group though it was thought to be produced by the botulinum strains only.

Forty four strains identified as Group III clostridia by phenotype were investigated for the distribution of toxin genes by PCR to confirm the identities of these organisms. Several combinations of the species-defining genes and the C2 genes were found. The reference *C. botulinum* types C and D strains and one non-neurotoxic strain of *C. botulinum* type C carried the genes for both components of the C2 toxin. Of the 40 *C. novyi* type A strains, the gene for the alpha toxin was found in 22 with 19 of these strains carrying the gene for one component of the C2 toxin. In the 18 alpha toxin-negative strains, 2 carried the genes for both components of the C2 toxin and a further 11 strains carried the gene for the component I only. The detection of the C2 toxin genes in strains identified as *C. novyi* type A is novel and may suggest a role for one or both components of the C2 toxin in disease.

Acknowledgements

I would like to thank my supervisors, Professor Ian Poxton and Professor David Smith for all of their help and encouragement during the course of this PhD. I would also like to thank David Smith and the Functional Genomics unit at the Moredun Institute for all of their help with the sequencing work. I would like to acknowledge Bob Brown, Fraser Pike and Allison Wroe for their assistance in the culture work.

I would like to thank Professor Bruce McGorum and Dr. Scot Pirie for their involvement in the Dubai study and for collecting samples. I would also like to thank Tom Pennycott from the SAC for his involvement in the work in botulism in sea birds and for sending me samples and Bernie Zonfrillo for collecting the Fulmar samples.

I would like to acknowledge the Dubai Millennium Research Foundation for funding my studentship.

Most of all I would like to thank my family and friends for all of their support and encouragement during the course of my PhD.

Declaration

All of the experiments carried out in this thesis were performed by the author unless otherwise indicated in the text.

A. Hoffman.

Abbreviations

Abbreviations used in this thesis

16S	16 Svedburg RNA
ADP	Adenosine diphosphate
AFLP	Amplified fragment length polymorphism
AGS	Acute grass sickness
AIM	Anaerobe identification medium
BoNT	Botulinum neurotoxin
BSA	Bovine serum albumin
CBA	Colombia blood agar
CGS	Chronic grass sickness
CMB	Cooked meat broth
CROPs	Clostridial repetitive oligopeptides
DIG	Digoxigenin
EBVC	Easter Bush veterinary centre
EGS	Equine Grass Sickness
ELISA	Enzyme linked immunosorbent assay
EYA	Egg-yolk agar
FAA	Fastidious anaerobe agar
FASTA	Fast-All
FITC	Fluorescein isothiocyanate
GAPs	GTPase-activating proteins
GDI	Guanidine nucleotide dissociation inhibitor
GDP	Guanidine diphosphate

GEFs	Guanidine nucleotide exchange factors
GI tract	Gastro-intestinal tract
GLC	Gas liquid chromatography
GTP	Guanidine triphosphate
HA	Haemagglutinin
HRP	Horseradish peroxidase
IDU	Injecting drug user
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IV	Intravenous
kDa	kilodalton
MPRL	Microbial Pathogenicity Research Laboratory
NAD	Nicotinamide adenine dinucleotide
NSF	N-ethylmaleimide sensitive fusion protein
NTNH	non-toxic, non-haemagglutinin
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Rho	Rhodopsin
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
Tet	Tetanus

Tn	Transposon
Tox	Toxin
UDP	Uracil diphosphate
VAMP	Vesicle associated membrane protein
VFA	Volatile fatty acid

Chapter One

Introduction

1.1 Clostridia

The clostridia comprise a group of gram positive, anaerobic spore forming bacilli. They are pleomorphic both in colony type and cell type with some strains exhibiting both swarming growth and discrete colonies on solid media. By microscopy they are typically seen as large, straight rods with curved ends but may also appear as elongated cells, filaments or club shaped cells. Clostridia show typical Gram positive cell envelope morphology but may appear Gram variable and can appear Gram negative, particularly in older cultures. All clostridia produce spores that may be oval or spherical and often distend the cells. Most species are obligate anaerobes though a degree of oxygen tolerance is exhibited by some. Sporulation is not thought to occur when the organisms are grown under aerobic conditions. They are ubiquitous in soil and the environment and some are found as commensals of the human and animal gut. The organisms may be saccharolytic and / or proteolytic. Clostridia characteristically ferment carbohydrates and peptides for energy production and the volatile and non-volatile fatty acids produced as the end product of this metabolic activity can be used to identify clostridia by gas liquid chromatography (GLC).

The species of clostridia that are pathogenic for humans and other animals typically produce one or more exotoxin.

1.2 *Clostridium botulinum*

1.2.1 Phenotypic and genotypic groups of *C. botulinum*

The *Clostridium botulinum* species is a collection of organisms that vary phenotypically and genotypically. They are classified as a single species on the basis of their ability to produce neurotoxins with similar pharmacological actions. The botulinum neurotoxin is one of the most potent naturally occurring neurotoxins.

C. botulinum has been divided into seven types, A to G, based on the antigenicity of the neurotoxin produced.

C. botulinum has been grouped into four groups based upon phenotypic characteristics. Group I contains Type A and proteolytic type B strains as well as the non-neurotoxigenic *C. sporogenes* which is phenotypically related. Group II contains non-proteolytic type B and type E strains and *C. perfringens*. Type C and D *C. botulinum* and *C. novyi* comprise the Group III organisms and Group IV consists of *C. botulinum* type G (though this has been renamed *C. argentinense*) and *C. subterminale*. The phenotypic characteristics of these four groups are given in Table 1.1.

Group	Type of toxin	Milk digestion	Glucose fermentation	Lipase	Lecithinase	Metabolic Products Volatile	Non-volatile
I	A, B, F	+	+	+	-	A, iB, B, iV	PP
II	B, E, F	-	+	+	+/-	A, B	
III	C, D	+/-	+	+	+	A, P, B	
IV	G	+	-	-	-	A, iB, B, iV	PA

Table 1.1: Phenotypic characteristics of the four clostridial groups. Metabolic end products are separated into volatile and non-volatile fatty acids. A, acetic; P, proprionic; iB, isobutyric; B, butyric; iV, isovaleric; PP, phenylproprionic; PA, phenylacetic.

The nomenclature of *C. botulinum* strains based upon toxin production is not straightforward. Though toxins A, B and E are chromosomally encoded and therefore stable, there have been strains isolated that are capable of producing combinations of two toxin types. Two strains were isolated from children with botulism that produced 90% type B toxin and 10% type F (Hatheway and McCroskey 1987) and a soil isolate was found to produce 93% type A and 7% type F (Hatheway 1989). The isolation of *Clostridium barati* that produces type F toxin (Hall et al. 1985) and *Clostridium butyricum* that produces type E toxin (McCroskey et al. 1986) and have been implicated in cases of botulism further complicates the classification of *C. botulinum.*

Genetic studies based on 16S ribosomal RNA have demonstrated four distinct groups that correlate with the groups based upon phenotypic characteristics (Hutson et al. 1993). Furthermore it has been demonstrated that all strains within Group I or Group II are closely related though a degree of genetic diversity exists between the organisms classed as Group III clostridia (Lee and Riemann 1970; Nakamura et al. 1983).

The type C and D toxin genes are carried on bacteriophages in an unstable association with the host cell and interconversion has been demonstrated within the Group III clostridia. Plasmids have been isolated from all groups of clostridia and include all toxin types (Strom et al. 1984). Multiple plasmids were found in types A and B (Group I clostridia) and in the Group III organisms *C. botulinum* type C and type D. There is thought to have been lateral transfer of the BoNT genes between the groups.

It has been suggested that *C. botulinum* should be reclassified into four separate species based upon the phenotypic and genotypic differences (Hutson et al. 1993).

1.2.2 The botulinum neurotoxins

Despite the antigenic differences between the botulinum neurotoxins they all have similar pharmacological activities. They inhibit the release of neurotransmitter at cholinergic synapses. The toxin is produced as a single chain molecule that is cleaved by bacterial or host proteases to form the active bipartite toxin consisting of a heavy chain (binding portion) covalently linked to the light chain (active component) by a disulphide bond. The light chain component is a zinc-dependent endopeptidase. The mechanism of action of the botulinum neurotoxins consists of four stages; binding to the neuronal membrane, internalisation, membrane translocation and enzymatic modification of the target in the cytosol (Montecucco et al. 1994).

The structure of the botulinum neurotoxins

Botulinum neurotoxins are produced in the form of macromolecular complexes termed progenitor toxins. These complexes range in size from 300-900kDa. The larger complexes (with sedimentation rates 19S and 16S) may include a haemagglutinin though the 19S form has only been associated with type A toxin. The type C and type D toxins form both 12S and 16S complexes. The 12S complex consists of the neurotoxin and a non-toxic non-haemagglutinin (NTNH) component. The 16S complex consists of the neurotoxin, NTNH and a haemagglutinin

component (HA) (Fujinaga et al. 1997). The progenitor toxin complex is thought to be important for the oral toxicity of the derivative toxin providing protection from proteases and acidity in the GI tract (Ohishi and Sakaguchi 1980).

The derivative toxin consists of a 100kDa heavy chain and a 50kDa light chain. The heavy chain is susceptible to the action of trypsin which cleaves it into two fragments, H_C at the carboxy end appears to possess the binding site and H_N at the amino end remains linked to the light chain and is thought to be involved in membrane translocation (Shone et al. 1985; Oguma et al. 1995).

Cell binding and internalisation

In order to bind to the pre-synaptic membrane at the neuromuscular junction the botulinum neurotoxin must first diffuse into the circulation from the site of production or adsorption. The most common route of entry is via the GI tract, either from ingestion of pre-formed toxin or the localised production of toxin.

Binding studies have shown that the HA component of the 16S type C progenitor is important in binding to glycoproteins and glycolipids in the small intestine in guinea pigs. No binding activity was demonstrated in either the type C neurotoxin or NTNH component. It is thought that this may account for the reduced oral toxicity of the 12S complex or purified neurotoxin alone. (Fujinaga et al. 1997). Both type C and D toxins bind to sialoglycolipids and sialoglycoproteins but not to neutral glycolipids or asialoglycoproteins (Inoue et al. 1999). The neurotoxin does not dissociate from the

16S complex in the small intestine and the toxin therefore remains anchored to the intestinal epithelium by the HA component (Fujinaga et al. 1997).

The botulinum neurotoxins are thought to cross the intestinal epithelium by transcytosis (Maksymowych and Simpson 1998). Type A and B toxins were found to be bound and transcytosed by human intestinal cells in a functionally active form. Type C toxin does not appear to be efficiently transcytosed by human epithelial cells which may explain why it is not associated with human disease though purified toxin alone was used; it is not known if the 16S complex is able to bind to human cells (Maksymowych and Simpson 1998).

The carboxy terminal of the heavy chain has been implicated in neurospecific binding (Schiavo et al. 2000). This C terminal is composed of two distinct regions; the amino terminal region is highly conserved between the botulinum neurotoxins. The carboxy terminal region is poorly conserved and is required for binding of the different types of toxins to separate receptors. A double receptor model for binding of clostridial neurotoxins to neuronal cells has been proposed to account for the actions of both polysialogangliosides and the glycoprotein receptor protein. The polysialoglycoside binds all neurotoxins, acting as a trap for them and enabling the small amounts of neurotoxin to cause disease. Bound toxin would then be able to interact with the specific glycoprotein receptor that is responsible for the neurospecificity of the toxins. Pre-incubation of botulinum neurotoxin with gangliosides has been shown to inhibit binding to varying degrees depending on the ganglioside. GT1b leads to a loss in toxicity of all toxin types (Kozaki et al. 1984).

The specific receptors for the different toxin types are not known though binding of BoNT/C is completely inhibited by pre-treatment of cells with neuraminidase, pronase or trypsin indicating that a glycoprotein containing sialic acid may act as the receptor (Yokosawa et al. 1989).

It has been proposed that internalisation of the neurotoxin is by receptor mediated endocytosis via clathrin coated pits (Deinhardt et al. 2006). Once in the cell, the toxin must be able to leave this compartment in order to reach the intracellular substrate. It is thought that at low pH the toxins undergo conformational changes leading to the exposure of hydrophobic segments enabling both the heavy and light chains to penetrate the hydrocarbon core of the vesicle membrane. The neurotoxins have been shown to form ion channels in planar lipid bilayers (Donovan and Middlebrook 1986).

Intracellular action

The botulinum neurotoxins are zinc-dependent metalloproteases. The Light chain contains a highly conserved central region with a His-Glu-X-X-His motif which forms the zinc binding region; zinc plays an important role in the hydrolysis of the peptide bond (Schiavo et al. 1993). They cleave the proteins involved in neuroexocytosis at a single specific site resulting in a sustained blockage of neurotransmitter release (Table 1.2)

SNAP-25 and Syntaxin are bound to the pre-synaptic membrane and Synaptobrevin (VAMP) is found on the vesicle membrane. These proteins form the SNARE (SNAP receptor) complex required for neuroexocytosis. The SNARE proteins (VAMP and

Syntaxin) promote membrane fusion and exocytosis together with the soluble factors NSF (N-ethylmaleimide sensitive fusion protein) and α, β and γ SNAPS (soluble NSF attachment proteins). The pre-formed complex is highly stable and is resistant to the action of the botulinum neurotoxins which prevent the formation of the SNARE complex (Hayashi et al. 1994) (Fig 1.1).

The botulinum neurotoxins are thought to recognise their substrates by a two-step process. It is thought that the initial recognition is by a nine amino acid SNARE motif common to all three targets (Schiavo et al. 2000) and by recognition of the specific peptide bond to be cleaved (Rossetto et al. 1994). This model accounts for the observation that only one of many identical peptide bonds within the target protein is cleaved and that short peptides containing the cleavage site are unaffected (Montecucco and Schiavo 1994).

Toxin type	Target
BoNT/A	SNAP-25
BoNT/B	VAMP
BoNT/C	Syntaxin, SNAP-25
BoNT/D	VAMP
BoNT/E	SNAP-25
BoNT/F	VAMP

Table 1.2: Botulinum neurotoxin targets (Schiavo and Montecucco, 1997)

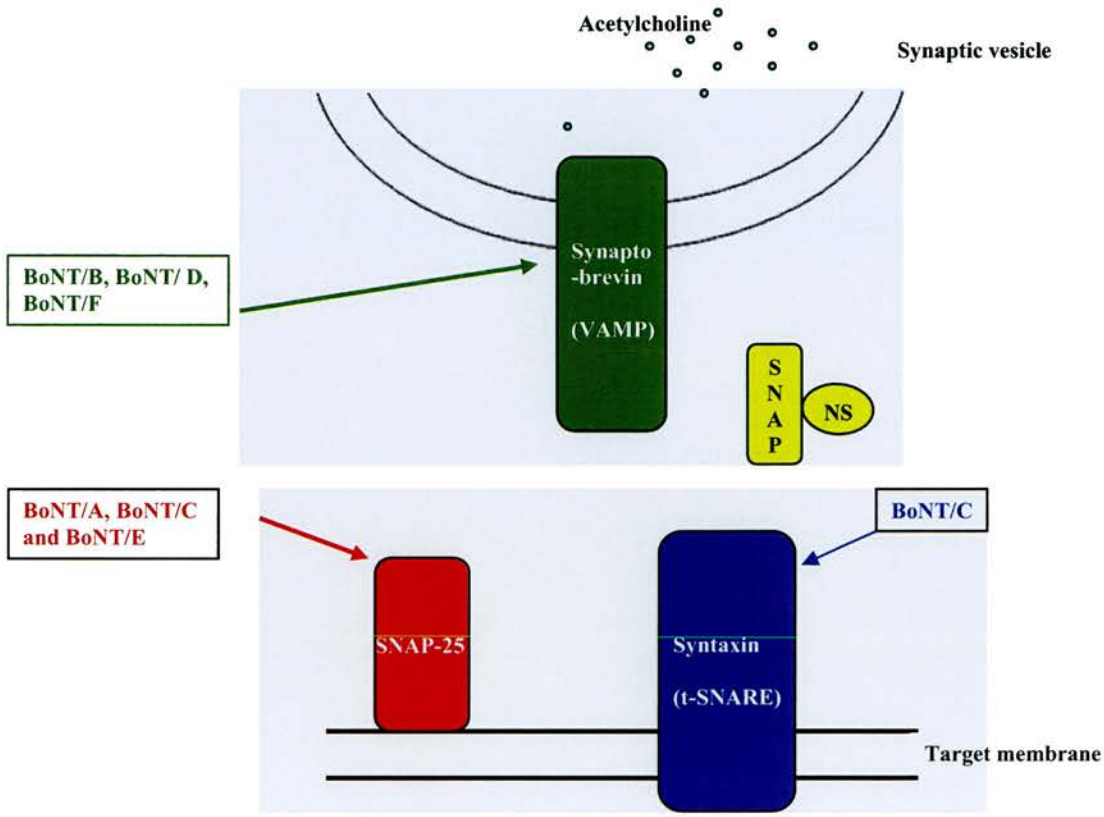

Figure 1.1: Schematic diagram of the SNAP/SNARE model of neuroexocytosis showing the targets of the botulinum neurotoxins. (Adapted from Oguma et al, 1995)

Cytotoxicity of the type C neurotoxin

In addition to its ability to block neurotransmitter release the type C toxin has been shown to be cytotoxic to murine spinal cord cells in vitro (Williamson et al. 1995) and to rat hippocampal cells and cortical neurons (OsenSand et al. 1996). The BoNT/C is the only neurotoxin capable of causing overt neuronal degeneration and affects both mature and developing cells in vitro (Williamson and Neale 1998).

Neuronal cell damage was observed in primary neuronal cell cultures following 48h incubation with BoNT/C with cells showing a loss of normal structures; accumulation of degenerated mitochondria, dense membranous bodies and vesicles. The neuronal somas were spherical and showed distorted nuclei and loss of Nissi bodies (Kurokawa et al. 1987). These ultrastructural changes are similar to retrograde degeneration of axons in vivo. In mature cells enlargement of the synaptic terminals is seen shortly after exposure to BoNT/C followed by degeneration of axons, dendrites and cell bodies (Williamson and Neale 1998).

The BoNT/C acts on syntaxin and is the only botulinum neurotoxin to do so. It is thought that the neuronal cell death is mediated by the effects of BoNT/C on syntaxin. It has been shown that syntaxin is required for neuronal cell development and survival (Kurokawa et al. 1987; Williamson et al. 1995). Botulinum neurotoxins can inhibit neurotransmitters other than acetylcholine in vitro due to the conservation of the intracellular targets within neuronal synapses (Sugiyama 1980; Williamson and Neale 1998). It is possible that BoNT/C is able to cause damage to a wide range of neurons in vivo though it has not been shown to cause loss of motor neurons in humans when used to treat dystonia (Eleopra et al. 1997).

1.2.3 Toxins and disease

There are three forms of botulism: classical botulism caused by the ingestion of pre-formed toxin and two forms of toxicoinfectious botulism resulting from the absorption of toxin produced in vivo. This toxin production may occur in the intestinal tract following colonisation with *C. botulinum* or, more rarely, in a wound. The main route of exposure to botulinum toxin is via the GI tract.

Types A, B, E and F botulinum are typically associated with human disease and types C and D are thought only to affect animals (Rocke, 1993). There have been reports of type C botulism in humans: two cases were reported in the USSR, one case in France and one in the USA (Jensen and Price, 1987). BoNT/C has been shown to be able to cleave recombinant human syntaxin and block the release of neurotransmitter from isolated human neuromuscular junctions (Coffield et al. 1997). It is therefore not known why *C. botulinum* type C rarely causes disease in humans though it may be due to a lack of exposure to the toxin. It has been proposed that the human GI tract lacks receptors for BoNT/C and therefore BoNT/C is unable to enter the circulation (Maksymowych and Simpson 1998).

Botulism is also seen in most domestic animals and avian species. Outbreaks of botulism occur most frequently in birds (Lamanna, 1987) and though birds are susceptible to all types of botulinum toxin, type C toxin is responsible for the majority of cases. Botulism has also been reported in a mink, ferrets, dogs, cats,

horses, cattle and pigs. Horses are more susceptible to botulism than species such as cattle, dogs and humans (Whitlock and Buckley 1997).

Foodborne botulism

Foodborne botulism is the most common form of the disease and is caused by the ingestion of pre-formed toxin that has been produced in the food. Botulinum toxin was first identified as the cause of disease in 1895 following an outbreak of botulism caused by salt-cured ham (van Ermengen, 1897 cited in Hatheway, 1989). Botulism in humans is rare and outbreaks are traditionally associated with consumption of home-canned or bottled vegetables or meat (Macdonald et al. 1986). A recent outbreak of botulism in Texas following a church supper was traced to a dish made from frozen chillies that had not been stored adequately. It is more common in Southern and Eastern Europe than in Northern and North-western Europe. Botulism in animals is more common, possibly due to the consumption of rotting carcases and other spoiled foodstuffs.

Rotting carcases of both vertebrates and invertebrates are a common source of botulism for animals and birds. In particular, maggots have been shown to contain high levels of BoNT/C (Haagsma, 1987) and provide a significant source of toxin for birds (Mitchell and Rosendal, 1987). *C. botulinum* is thought to invade the tissues from the gut following death. Mice were inoculated with type C spores immediately prior to death, and the carcases incubated at 28°C. High levels of BoNT/C were detected in these carcases for 28 days (Smith and Turner 1989).

Landfill sites are also thought to be a source of botulism for herring gulls due in part to their scavenging feeding habits. Spores of *C. botulinum* types B, C and D were found in 63% of landfill sites (Ortiz and Smith 1994). The origin of the spores is not known though carriage of *C. botulinum* spores has been documented in the alimentary tracts of gulls.

Botulism in domestic animals is often referred to as forage poisoning and is associated with the consumption of contaminated feed. Forage poisoning is usually associated with type C botulinum in Northern Europe and type B in North America.

Toxicoinfectious botulism

The most common form of toxicoinfectious botulism is infant botulism, caused by the production of toxin in the GI tract. Spores of *C. botulinum* colonise the immature gut of children typically under 1 year old where they germinate and produce toxin. The toxin is then absorbed through the gut wall and spreads systemically resulting in a flaccid paralysis. Infant botulism is rare in many countries though it is now the most common form of botulism in the US (Hatheway 1990). Honey contaminated with spores of *C. botulinum* type B has been implicated in many cases of infant botulism the US (Arnon 1980).

Intestinal colonisation with *C. botulinum* has been reported in adults but is extremely rare and typically follows disruption to the GI flora following surgery or antibiotic use (Chia et al. 1986). *C. botulinum* does not form part of the normal flora of the human GI tract. Following intestinal colonisation of both adults and infants the

presence of both the organism and toxin in the faeces has been demonstrated after recovery from the disease (McCroskey and Hatheway 1988).

'Shaker foal syndrome' is usually caused by type B botulinum and affects foals between 2 weeks and 8 months of age. It is thought that spores are ingested from the soil and are able to colonise the immature gut. Toxin is produced in necrotic lesions in the GI tract. Toxin is only detected in the faeces during the acute phase of the disease. Toxicoinfectious botulism has not been documented in adult horses (Whitlock and Buckley 1997).

Toxicoinfectious botulism caused by type C botulinum is seen in outbreaks of botulism in broiler chickens and it is thought that the high-energy diet enhances both the rate and amount of toxin production in the GI tract (Eklund et al, 1987). Toxigenic and non-toxigenic strains of *C. botulinum* type C have been isolated from poultry litter and this is thought to be a source of infection for the chickens (Eklund et al, 1987). Poultry litter that is fed to cattle or used to fertilise fields has been associated with botulism in cattle (McLoughlin et al. 1988; Ortolani et al. 1997).

Wound botulism

Wound botulism was first identified in 1943 with the isolation of *C. botulinum* type A from the wound of a patient who had died of flaccid paralysis (Hatheway 1990). Wound botulism is the least common form of botulism with only 16 cases reported between 1967 and 1984 in the US and no cases reported to the Public Health Laboratory Service of England and Wales prior to 1999. Wound botulism is

increasing in the UK (England and Wales) with five cases reported in 2000, four in 2001, twenty in 2002, fifteen in 2003, forty in 2004, twenty-eight in 2005 and 17 in 2006 (http://www.hpa.org.uk/). All cases were associated with IV drug abuse and the practise of skin or muscle popping. The clinical symptoms of wound botulism are the same as those seen in the classical form of the disease with neurotoxin produced in the wound site entering the circulation.

1.3 Equine Grass Sickness (EGS)

Equine grass sickness is a largely fatal primary dysautonomia affecting horses and other equids. Its aetiology is unknown. It was first recognised in 1909 in army horses in Forfar, Scotland. The disease occurs mainly in the UK with a higher incidence in Scotland (Milne et al. 1994) but has also been reported in Northern Europe, Scandinavia (Gilmour 1987) and Australia (Stewart 1977). EGS is thought to be identical to 'mal seco', a dysautonomia occurring in Argentina and Chile (Uzal and Robles 1993).

1.3.1 Clinical signs

Equine grass sickness occurs in three forms, acute, subacute and chronic based upon the severity of clinical signs and duration of symptoms (Doxey et al. 1991). Acute grass sickness is the most severe form of the disease with a sudden onset of symptoms and death or euthanasia occurring within 24 hours of the onset of symptoms. Subacute grass sickness also has a rapid onset on symptoms though horses may survive for up to seven days. Chronic grass sickness has a slower onset of symptoms which can last for months before recovery of the horse or death.

EGS is characterised by dysfunction of the gastrointestinal tract. Clinical signs range from inability to swallow (dysphagia) and varying degrees of GI stasis. In the acute from complete GI stasis with impaction of the colon and accumulation of fluid in the small intestine may be seen. The clinical signs are thought to reflect the extent of neuronal damage. The symptoms are less severe in subacute grass sickness with reduced gut motility seen. The chronic form of the disease is characterised by a rapid loss of weight and the development of a tucked up abdomen. Other symptoms include a narrow base stance, muscle tremors, patchy sweating and ptosis (drooping of the eyelid). There is no single pathognomonic sign for grass sickness and diagnosis can only be confirmed by post-mortem detection of the neuronal degeneration of peripheral autonomic ganglia (Doxey et al. 1995).

There is currently no treatment for acute and subacute grass sickness though some chronic cases can recover with intensive nursing. These cases are typically those with milder symptoms, in particular those involving dysphagia, loss of appetite, colic and reduced gut motility (Milne et al. 1994).

Equine grass sickness is associated with severe neuronal degeneration and loss in the autonomic and enteric nervous systems. Neuronal lesions were first observed in the vertebral and prevertebral ganglia and the alimentary mural plexei (Obel, 1955). Neuronal lesions have since been demonstrated in the central nervous system in the brainstem nuclei, the dorsal root ganglia, the spinal cord, the intermediolateral nucleus and the ventral horn (Barlow 1969; Gilmour 1973). Neuronal degeneration is

more widespread in the autonomic nervous system than in the central nervous system. Enteric cholinergic neurons from the ileum of horses with grass sickness showed altered cholinergic mechanisms and a reduction the release of acetylcholine (Murray et al. 1994).

Neuronal degeneration is thought to be the primary event in grass sickness (Doxey et al. 1995). The pattern of neuronal degeneration is indicative of a toxic cause. The clinical severity of the disease reflects the extent of neuronal damage (Scholes et al. 1993). In acute grass sickness, neuronal degeneration is observed at most sites along the GI tract whereas in chronic grass sickness it is restricted to the terminal small intestine, in particular the ileum (Scholes et al. 1993; Doxey et al. 1995). It has been hypothesised that equine grass sickness may be caused by a toxin that is either ingested or produced within the GI tract (Doxey et al. 1995) and that the ileum is the likely site of entry or toxin production given the extensive damage to the ileum irrespective of the severity of disease (Scholes et al. 1993; Doxey et al. 1995). The dosage of toxin and duration of exposure would therefore account for the differences in the disease with acute cases exposed to large amounts of toxin along the length of the GI tract over a short period of time. Chronic cases have less neuronal damage which could be due to exposure to lower doses of the toxin in the distal ileum only (Doxey et al. 1995). Damage to the peripheral autonomic nervous system could be caused by toxin entering the circulation (Doxey et al. 1995) or by retrograde axonal transport (Griffiths et al. 1994). Severe neuronal degeneration with little cell loss is a characteristic of the acute form of the disease (Pogson et al. 1992) whereas horses

with chronic grass sickness are typically found to have fewer damaged neurons but show reduced counts of neurons (Pogson et al. 1992).

Epidemiology

Grass sickness, as the name suggests is associated with grazing. This was recognised when the disease was first discovered (Tocher et al, 1923) and has been subsequently confirmed in several epidemiological studies (Gilmour and Jolly 1974; Doxey et al. 1992; Wood et al. 1998). The disease shows a seasonal distribution with the majority of cases in the UK occurring between April and July (Doxey et al. 1991). The disease is more likely to occur on premises where grass sickness has previously occurred, particularly in the last two years (Wood et al. 1998), horses that have recently moved premises or to a different grazing area show an increased risk (McCarthy et al. 2004a). Young horses are more at risk then older animals with the peak age range for disease being 1-7 years old (Gilmour and Jolly 1974). Prior contact of a horse with a case of grass sickness has been associated with a reduction in the risk of disease (Wood et al. 1998) though it is not known if this represents inherent resistance to the disease or if non-fatal exposure to the disease confers resistance.

Aetiology

The epidemiology of the disease, in particular the seasonal distribution, difference in susceptibility depending on age and the low incidence of the disease in horses with prior contact with cases suggests an infectious aetiology with the causal agent related to grazing.

Several factors have been suggested as putative aetiological agents including poisonous plants, in particular white clover, fungi including *Fusarium* species and oxidative stress in horses due to the consumption of plants under metabolic stress (McGorum et al. 2000). There is little evidence to support these and many have been discounted. A toxin producing bacterium seems to be the most likely cause.

In 1919 *Clostridium botulinum* was isolated from the stomach contents and intestine from a horse with grass sickness (Tocher et al, 1923) and similarities between grass sickness and classical botulism were noted and *C. botulinum* was considered to be the cause of the disease. The organism was isolated from the spleens of horses with subacute grass sickness and the isolated organism was said to reproduce the symptoms of the disease in experimental animals (Tocher et al, 1923). The work of Tocher was highly criticised and the theory was largely discounted. In particular the protection study carried out by Tocher using a toxin/antitoxin mixture prepared from known strains that reduced mortality in vaccinated animals to 2.8% compared to 9.3% in un-vaccinated controls (Tocher et al, 1923). This was criticised on the grounds that human strains had been used for the preparation of the toxin/antitoxin mixture and not those isolated from horses There was also criticism that the organism isolated from horses had not been conclusively identified as *Clostridium botulinum* or isolated in pure culture (Gaiger in Tocher et al, 1923). Gaiger, who had proposed that grass sickness was cause by streptococci, was then appointed as the Chief Investigator of the Animal Diseases Research Association and took on the organisation of grass sickness research. The theory proposed by Tocher re-emerged in 1994 when it was hypothesised that grass sickness is caused by a toxicoinfection

with *C. botulinum* type C (J.K.Miller, pers. comm.). The BoNT/C has been detected in 74% of acute cases, 67% of subacute and chronic cases and only 10% of control cases from faecal or ileal samples (Hunter, 2000). Significantly lower levels of antibodies (IgG) to the surface antigens of the closely related *C. novyi* type A and to the BoNT/C were detected in horses with grass sickness compared to controls (Hunter and Poxton 2001). Grass sickness has also been shown to be significantly associated with low antibody levels to the surface antigens of *C. novyi* type A, *C. botulinum* type C and BoNT/C in a matched case-control study (McCarthy et al. 2004b).

1.4 Group III clostridia

C. botulinum type C and D and *C. novyi* type A are grouped together on the basis of their phenotypic and genotypic properties. *C. novyi* type A is a non-neurotoxigenic variant of Group III botulinum. These organisms cannot be distinguished from each other using traditional methods such as culture, biochemical properties or GLC profiles. Their surface antigens are immunologically cross-reactive (Poxton 1984; Poxton and Byrne 1984).

Group III organisms can only be identified to species level by the detection of the major toxin produced; type C and D neurotoxin or the novyi alpha toxin. These toxins are encoded on separate pseudolysogenic bacteriophages. The phage-host relationship is unstable and the phages are readily lost. Cycles of phage loss and reinfection are thought to occur in vivo (Eklund and Poysky, 1974). Repeated subculturing and growth at 37°C can predispose to loss of the phage. Non-toxigenic Group III organisms are indistinguishable from each other.

Sequence analysis of the 16S rRNA gene grouped *C. botulinum* type C and D and *C. novyi* type A as a separate phylogenetic lineage (Hutson et al. 1993). There is sequence diversity within this group however. There is 99% sequence homology between type C and D botulinum which corresponds to 12 base mismatches and seven unmatched bases over 1500 nucleotides (Hutson et al. 1993). The 1% sequence divergence suggests that *C. botulinum* type C and D are separate species based on genetic heterogeneity (Collins and East 1998). *C. novyi* shows 98% sequence

similarity based on the 16S rRNA gene sequence (Hutson et al. 1993). The 2% difference is also considered sufficient for *C. novyi* to be classed as a separate species.

C. novyi type A strains are commonly associated with wound and soft tissue infections causing gas gangrene in humans. It was typically found in wounds contaminated with soil e.g. war wounds. *C. novyi* infections had been extremely rare until it re-emerged in 2000 as the cause of wound infection in injecting drug users (IDUs).

1.4.1 Bacteriophages and Group III clostridia

Bacteriophages have been found in Groups I, II, and III clostridia, but have only been associated with toxigenicity in the Group III clostridia (Eklund et al, 1989). Production of the major toxins is governed by the presence of the specific converting bacteriophages (Eklund et al. 1971; Eklund et al. 1972; Eklund et al. 1976) Loss of the bacteriophage is associated with a stable and permanent loss of toxicity. Non-toxigenic isolates of toxigenic parent strains and isolates that had lost their bacteriophage could be re-infected with the converting phage restoring toxigenicity to the isolate.

The converting phages are thought to be pseudolysogenic due to the instability of the host-phage relationship. Non-toxigenic isolates resembling *C. botulinum* have been isolated from environmental samples and subculturing of toxigenic isolates leads to a loss in toxigenicity (Eklund et al, 1987). This suggests that the phage is not stably integrated into the host cell chromosome. Additionally, *C. botulinum* type C and D

can be cured of their converting phages using both ultraviolet irradiation and acridine orange (Eklund et al. 1971). Ultraviolet irradiation is a standard method of curing cells of lysogenic bacteriophages and acridine orange is used to remove extrachromosomal genetic elements from cells. The BoNT/C converting phage has been shown to exist as a circular plasmid prophage in the lysogen rather than integrating into the host chromosome (Sakaguchi et al. 2005). Subculture of toxigenic isolates in the presence of phage-specific antiserum also results in loss of the bacteriophage from the cell. Although this occurs spontaneously during sub-culture, a higher number of non-toxigenic isolates are found in the presence of anti-phage serum (Oguma et al. 1976). This suggests that non-toxigenic strains can be re-infected by free phages in the culture medium.

Interconversion of *C. botulinum* type C and D

C. botulinum type C and D have been shown to be interconvertible by bacteriophages (Eklund and Poysky 1974). Loss of the converting phage from the bacteria leads to strains that can not be identified to species level. The non-toxigenic strains of *C. botulinum* type C can be re-infected with the bacteriophage from a type D strain resulting in the production of the type D neurotoxin. Similarly, non-toxigenic strains of *C. botulinum* type D can be re-infected with the bacteriophage from a type C strain resulting in the production of the type C neurotoxin.

The C2 toxin is a binary ADP-ribosylating toxin produced by the majority of type C and some type D strains. It is chromosomally encoded and is produced during sporulation (Nakamura et al. 1978).

In non-C2 producing strains, type C strains that had been cured of their converting phages could be infected by either the type C or type D phage but the type D strains could only be re-infected with the homologous phage (Eklund and Poysky 1974). Cultures infected with the type C phage were immune to infection with the type D phage and vice versa suggesting that the phages are antigenically related (Eklund et al, 1974).

It has been suggested that interconversion between type C and D can occur in nature as a result of the pseudolysogenic nature of the bacteriophages and that types C and D can arise from a common non-toxigenic strain. The genetic variation based on 16S rRNA sequence that suggests these organisms are separate species was based on comparison between one type C and one type D strain. Genetic heterogeneity has been demonstrated within *C. botulinum* type C strains (Nakamura et al. 1983).

Interconversion between *C. botulinum* and *C. novyi*

Loss of the phages carrying the type C and D neurotoxins and the novyi alpha toxin leads to isolates that are indistinguishable. Interspecies conversion between *C. novyi* and *C. botulinum* by infection with the relevant converting phage has been demonstrated (Eklund and Poysky 1974; Eklund et al. 1974). A non-toxigenic strain derived from *C. botulinum* type C cured of the type C phage could be infected with the phage from a *C. novyi* isolates resulting in the production of the novyi alpha toxin. Isolates infected with either the type C or type D phage are immune to infection with the type C and D phages but remain sensitive to infection with the novyi phage. Both

phages were shown to be carried through the spore state though one of the phages was frequently lost upon subculture (Eklund et al, 1987).

Antigenicity and stability of the bacteriophages

The type C and D phages are thought to form a phage family (Sunagawa and Inoue 1992). They share similar biological and biophysical properties and have a close DNA homology. They have also been divided into four groups based on the antigenicity of the phage and the spectrum of host cells they infect (Sunagawa and Inoue 1991). Conversion of a cell to toxicity only occurs with specific phages and bacterial cells. Phages have been isolated from *C. botulinum* type C and D and *C. novyi* type A that do not convert non-toxigenic isolates to toxin production (Sunagawa and Inoue 1991; Eklund et al, 1987). The non-converting phages are identical in their antigenicity and host specificity though they are smaller than the converting phages (Sunagawa and Inoue 1991).

Bacterial strains may differ in the stability of toxin production due to instabilities of the pseudolysogenic phages. Loss of toxigenicity occurs at different rates depending on the combination of phage and host (Oguma et al. 1976). Toxigenicity may also vary with the passage history of the phage in the laboratory. The converting ability of phages has been shown to decrease following repeated propagation through a bacterial strain (Oguma and Iida 1979). Incubation at 37°C was shown to select virulent mutants of the converting phages that were unable to pseudolysogenise the bacterial host and instead induced lysis of the host cell (Eklund et al, 1987).

The stability of the phage-host relationship may be influenced by environmental conditions. This is thought to affect the epidemiology of disease such as avian botulism cause by type C botulinum (Hariharan and Mitchell 1976). Avian botulism outbreaks are rare in areas with high alkalinity and are commonly seen in areas of moderate to low alkalinity such as marshlands (Eklund et al, 1987). High salt concentrations have been shown to inhibit the growth of *C. botulinum* type C (Eklund et al, 1987) whereas low salt concentrations increase both the rate of infection with the type C phage and the stability of the host-phage relationship (Eklund and Poysky 1974). This may account for the high incidence of *C. botulinum* type C at the periphery of salt flats and the association of these areas with avian botulism (Eklund et al, 1987).

1.4.2 Toxin production by Group III clostridia

Toxins produced by *C. botulinum* types C and D

C. botulinum type C and D are capable of producing up to three toxins. The defining neurotoxin, the C2 toxin (a binary ADP-ribosylating toxin) and the C3 toxin, an exoenzyme also associated with ADP-ribosylation. The C3 toxin is encoded on the converting phage with the BoNT/C or BoNT/D.

The C2 toxin is located on the bacterial chromosome. It is produced during sporulation only, with higher levels found in cultures producing high numbers of spores (Nakamura et al. 1978). It is thought that the C2 toxin may form part of the

spore coat as a structural protein (Yamakawa et al. 1983). It is produced by the majority of type C and some type D strains.

The type C and type D neurotoxins are closely related with 52.2 % sequence identity (Sunagawa et al. 1992). The toxins have common antigenic sites as well as antigen sites specific to the toxin type. Heterogeneity is seen in the type C and type D neurotoxins and is thought to be a result of mutation or recombination of the phage genome during the cycles of loss and re-infection with bacteriophages that occur naturally (Sunagawa and Inoue 1991).

The C3 exoenzyme has been divided into two groups on the basis of its antigenicity (Moriishi et al. 1993). It is thought that the 21.5kbp DNA fragment encoding the C3 exoenzyme may be a mobile genetic element (Hauser et al. 1995) due to its similarities with the site-specific transposon family Tn554.

Toxins produced by *C. novyi* type A.

C. novyi type A produces the species-defining novyi alpha toxin. The alpha toxin is one of a family of large clostridial cytotoxins. It is a lethal necrotising toxin in vivo causing oedema. The alpha toxin modifies GTP-binding proteins in vitro and is a potent cytotoxin. *C. novyi* type A also produces the gamma toxin, delta and epsilon toxins. The gamma toxin is a phospholipase C. The delta toxin is an oxygen-labile haemolysin and the epsilon toxin is a lipase. These toxins are responsible for the cultural characteristics of the organism; the gamma toxin produces lecithinase reaction observed in the culture of the organism on egg yolk agar and the epsilon

toxin is responsible for the 'pearly layer' associated with growth on egg yolk agar. The delta toxin gives the haemolysis associated with growth of the organism on blood agar.

1.4.3 Toxins that affect the cytoskeleton

The *C. novyi* alpha toxin, C2 toxin and C3 exoenzyme all affect the cytoskeleton causing a characteristic 'rounding up' of cells in vitro.

C. novyi alpha toxin

The *C. novyi* alpha toxin is one of a family of large clostridial cytotoxins including *Clostridium difficile* toxins A and B and the lethal toxin from *Clostridium sordelli*. These toxins are high molecular weigh (250-300kDa) molecules that glycosylate the low molecular weight GTP proteins of the Rho family (Busch and Aktories, 2000). The novyi alpha toxin is thought to consist of three functional domains: The N-terminal catalytic region, a central hydrophobic region involved in membrane translocation and a C-terminal binding region (vonEichelStreiber et al. 1996).

Cell binding and internalisation

Clostridial repetitive oligopeptides (CROPs) have been identified in the C-terminal region of the large clostridial cytotoxins (Hofmann et al. 1995). These consist of 25-30 amino acids repeated 14-30 times which function as ligands. They form multivalent receptor binding domains which enable multiple interactions with a receptor (vonEichelStreiber et al. 1996). The receptor for the novyi alpha toxin is unknown though it is thought to act ubiquitously against mammalian cells (Ball et al. 1993) suggesting the presence of a ubiquitous receptor.

The novyi alpha toxin is taken up into cells by receptor-mediated endocytosis. Acidification of the endosome is thought to induce a conformational change in the protein allowing entry into the cytosol (Ball et al. 1993). Translocation into the cytosol is thought to involve the insertion of the central hydrophobic region of the toxin into the endosomal membrane (vonEichelStreiber et al. 1996).

Intracellular action

The novyi alpha toxin acts on the proteins Rho, Rac and Cdc42 (Selzer et al. 1996) modifying them in their effector regions. The Rho-GTPases regulate the actin cytoskeleton and have a role in signal transduction (Hall 1998; Bishop and Hall 2000). The primary function of the Rho family is the regulation of the organisation of the actin cytoskeleton. Rho is involved in the formation of stress fibres, Rac is involved in the formation of membrane ruffles, lamellipodia and focal contacts and Cdc42 is involved in the formation of filopodia. Rho proteins also have a role in signal transduction cascades acting as molecular switches through nucleotide exchange in the GTPase cycle (Bishop and Hall 2000).

This cycle is tightly regulated. In its inactive form Rho is found bound to GDP in the cytosol. This inactive form is maintained by binding to guanidine nucleotide dissociation inhibitors (GDI). Rho is activated by GDP-GTP exchange mediated by guanidine nucleotide exchange factors (GEFs). Binding of GTP enables protein interaction with effector molecules. Hydrolysis of GTP to GDP by GTPase-activating proteins (GAPs) returns Rho to its inactive state.

The novyi alpha toxin modifies Rho at a specific amino acid (threonine 37) and Ras and Cdc42 at the homologous threonine 35 when Rho is in its inactive, GDP bound form (Selzer et al. 1996). This threonine residue is highly conserved and is involved in the binding of GDP/GTP to the Rho proteins. The alpha toxin acts as a catalyst in the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to Thr 37/35. The novyi alpha toxin is the only large clostridial toxin to use UDP-N-acetylglucosamine as a substrate rather then UDP-glucose (Busch et al. 2000). The glycosylation of Rho blocks the coupling of Rho proteins with their effector molecules and inhibits nucleotide exchange and membrane cycling (Sehr et al. 1998; Busch and Aktories 2000).

C2 toxin

The C2 toxin belongs to a class of actin-specific ADP-ribosyltransferases that includes the *Clostridium perfringens* iota toxin and the *Clostridium difficile* ADP-ribosylating toxin. The C2 toxin is a binary toxin composed of two separate components C2CI and C2CII (Aktories et al, 1992). Both components are required for toxicity. C2CI is the enzymatic component and C2CII is responsible for binding activity.

Cell binding and internalisation

The N-terminal region of C2CII is cleaved by trypsin to give an active form of the protein that forms oligomers, predominantly heptamers (Barth et al. 2000). The C-

terminal region is involved in binding to the cell surface (Blocker et al. 2000). Binding of C2CII was blocked by the use of an antibody against the C-terminal region and by truncation of this region. It is thought that the receptor binding site may be stabilised by the seven terminal amino acid residues as deletion of these also blocks binding (Blocker et al. 2000).

Activated C2CII binds to asparagine-linked complex carbohydrates (Eckhardt et al. 2000). This was determined using a C2 resistant cell line that was found to be deficient in N-acetylglucosaminyltransferase I. These cells were unable to make N-linked complex carbohydrates. Restoration of N-acetylglucosaminyltransferase I by gene transfection restored sensitivity to the C2 toxin (Eckhardt et al. 2000). There are no sequence similarities in the C-terminal region of the C2 toxin and other ADP-ribosylating toxins suggesting that the toxins act through different receptors. There is evidence that the C2 toxin acts through a carbohydrate receptor alone as no naturally resistant cell line has been found suggesting that the receptor is ubiquitous on mammalian cells (Eckhardt et al. 2000).

The N-terminal region of the C2CI component is involved in the interaction of the two components. The docking site is formed after binding of the C2CII component to the cell surface (Barth et al. 1998). The C2 toxin then enters cells by receptor mediated endocytosis (Simpson, 1989). Acidification of the endosomal compartment is required for entry into the cytosol (Barth et al. 2000). The C2CII has the ability to form ion channels in artificial lipid bilayers (Schmid et al. 1994) and it is thought

that following acidification of the endosome, oligomeric C2CII forms a channel in the membrane through which C2CI enters the cytosol (Barth et al. 2000).

Intracellular action

The C2CI component mono-ADP-ribosylates G actin at arginine 177 resulting in inhibition of actin polymerisation (Aktories et al. 1986). Actin forms an essential component of the cytoskeleton and is involved in motile processes such as migration, phagocytosis, secretion and intracellular transport based on its ability to polymerise and form microfilaments (Aktories and Wegner 1992). Actin exists in equilibrium between two forms; the filamentous F-actin and monomeric globular G-actin (Aktories and Wegner 1992). The ADP-ribosylated G-actin acts as a capping protein at the end of actin filaments preventing further polymerisation. Depolymerisation occurs at the opposite end.

The catalytic site is found at the C-terminal region with conserved glutamic acid residues at positions 387 and 389, serine-348 and arginine-299 essential for ADP-ribosyltransferase activity (Barth et al. 1998). A highly conserved catalytic glutamate residue is found in other ADP-ribosyltransferases (Barth et al. 1998). Serine-348 is thought to be involved in either NAD binding or as a catalyst and arginine-299 is thought to be involved in NAD binding or stabilising the active site.

1.4.4 Toxins and disease

C. novyi alpha toxin

The novyi alpha toxin is associated with soft tissue wounds in man and animals classically causing gas gangrene in war wounds. Gas gangrene is an invasive infection of the muscles characterised by toxaemia, localised oedema, tissue death and a variable degree of gas production (Maclennan 1962). Gas gangrene is associated with a variety of pathogenic clostridia and approximately one third of cases are associated with *C. novyi* type A (Smith, 1975). Wound infections involving *C. novyi* type A are associated with high levels of mortality due to the lethal necrotising properties of the alpha toxin.

C. novyi wound infections were considered rare until an outbreak of *C. novyi* wound infection among IDUs in 2000. Between April and August 2000, 60 IDUs in Scotland presented with severe skin and muscle damage with swelling at an intramuscular injection site with 23 patients dying of systemic shock. Cases were categorised as definite where both injection site inflammation and multi-system failure were seen and probable if either injection site inflammation or multi-system failure were seen. *C. novyi* was isolated from a total of 13 cases, 8 from definite cases and 5 from probable cases (McGuigan et al. 2002). *C. novyi* was the most frequently isolated pathogen and the clinical findings are consistent with the pathogenesis of *C. novyi*. The majority of cases had injected heroin extravascularly (muscle- or skin- popping). The source of the infection appeared to be a batch of heroin contaminated with spores of *C. novyi* type A. The heroin is thought to have been purer than normal (60-80% purity rather than the normal 30-40%) requiring the

addition of more citric acid in order to dissolve it for injection (McGuigan et al. 2002). The acidic solution is thought to have devitalised the soft tissue forming the site for growth and toxin production by *C. novyi.* Epidemiological studies revealed temporally related cases in England and the Republic of Ireland.

Amplified fragment length polymorphism (AFLP) analysis of *C. novyi* isolates obtained from the 2000 outbreak found the isolates from IDUs to be similar suggesting a common source (McLauchlin et al. 2002).

C2 toxin

The C2 toxin is able to cause morbidity and mortality in experimental animals though its role in natural infection is poorly understood. The C2 toxin can cause an increase in vascular permeability and fluid accumulation in intestinal loops (Ohishi 1983). Damage to the epithelial surface of the intestine is also seen (Ohishi and Odagiri 1984). The C2 toxin can also cause hypotension, haemorrhaging and a collection of fluid in the thoracic cavity in experimental animals (Simpson 1982).

The C2 toxin may have an effect on the immune system. It has been shown to inhibit neutrophil migration (Aktories et al, 1992) and to enhance granule protein release and the production of oxygen free radicals in neutrophils (Norgauer et al. 1988) and to inhibit histamine release from mast cells (Aktories and Wegner, 1989).

The C2 toxin has been implicated in disease in some cases of type C botulism in broiler chickens involving diarrhoea and enteritis in addition to the neurological symptoms (Ohishi and DasGupta, 1987). The C2 toxin has also been isolated from an

outbreak of botulism in horses in North America associated with the consumption of processed alfalfa hay cubes (Kinde et al. 1991). Intramuscular oedema was observed in five of the horses involved in the outbreak and suggests the possible involvement of the C2 toxin.

1.5 Clostridium tetani

C. tetani is a strict anaerobe. Most strains are motile by peritrichous flagella and exhibit a fine swarming growth an agar. Discrete colonies may be seen on media containing 3-4% agar. Colonies are flat and translucent showing a zone of beta-haemolysis. Spores are typically round and terminal. Their diameter can be up to twice that of the vegetative cell giving the characteristic 'drumstick' appearance.

1.5.1 Tetanus toxin

Tetanus toxin is produced as a single polypeptide chain of approximately 150kDa (Bizzini 1979). The molecule is subsequently cleaved into two fragments by proteases produced by the bacteria (Helting et al. 1979), a 100kDa heavy chain and a 50kDa light chain that are linked by a disulphide bond. Treatment of the bichain with papain cleaves the heavy chain resulting in two fragments; fragment B, a bichain molecule of 95kDa and fragment C, a 45Kda molecule derived from the heavy chain (Helting et al. 1979). These fragments are non-toxic.

The binding site is found on the heavy chain in the fragment C region and the high affinity of the toxin molecule and the fragment C portion for gangliosides suggests that the membrane receptors contain ganglioside structures (Morris et al. 1980). It has been proposed that a second, protein, receptor may also be involved in the binding of tetanus toxin (Montecucco 1986).

Tetanus toxin acts in the same way as the botulinum neurotoxins. It is a zinc dependent endopeptidase and cleaves VAMP. Tetanus toxin inhibits exocytosis in inhibitory neurons preventing the release of the inhibitory neuromuscular transmitters gamma-aminobutyric acid and glycine whereas the botulinum neurotoxins block neurotransmitter release at the neuromuscular junction. Though the mechanisms of action of the toxins are identical at the cellular level the differences in the site of action accounts for the differing symptoms with tetanus causing the characteristic spastic paralysis of the voluntary muscles and botulism resulting in a flaccid paralysis.

1.5.2 Tetanus toxin in disease

Classical tetanus was first described in ancient times and was first confirmed as a transmissible disease in 1884 (Hatheway 1990). The disease results from spores of *C. tetani* entering a wound. If the redox potential is low enough the spores germinate and growth of the organism occurs. This is typically seen in deep puncture wounds contaminated with soil. *C. tetani* is found in the GI tracts of many animals and is ubiquitous in soils. The tetanus toxin is produced in the wound and enters the system by either diffusion into surrounding muscle, by transport through the lymphatic

system or by passage through the nerve cells. The toxin passes through the nerve axons by retrograde transport to the central nervous system.

Clinical symptoms usually appear between 7 and 10 days after the wound becomes infected though they can appear between 3 and 30 days. Initial symptoms often include pain and stiffness, particularly around the wound site and trismus – where the muscles of the jaw are affected. Symptoms become generalised. Tetanus may be mild, with good response to treatment and low mortality, severe with 20-40% fatality or very severe with a 50-90% mortality rate. Tetanus is rare in the developed world due to an effective toxoid vaccine. Neonatal tetanus is still prevalent in some areas of the world with an estimated 400, 000 cases per year and is due to infection of the umbilicus, often associated with unusual anointing practices.

1.5.3 Neonatal tetanus and St Kilda

The Islands of St. Kilda form the most remote part of the British Isles and lie 110 miles west of mainland Scotland. The one inhabited island was largely self-sufficient with an economy based on seabirds. On 29th August, 1930 the Island was evacuated largely due to the decline in the population. Neonatal tetanus was common on St Kilda in the latter part of the 19th Century. It was referred to by the Islanders as 'the sickness of eight days'. Between 1855 and 1876 there were 56 births on the Island with 41 of the babies dying from neonatal tetanus. The source of this is unknown; there was a midwife on the island but midwifery procedures were highly secretive. It is thought that umbilicus was anointed with fulmar oil and this was the source of the infection (Woody and Ross 1989). Fulmars produce thick, orange/red oil as a by-

product of their diet of fish and are able to 'spit' this oil 2-3 feet as a defence against predators. The oil was collected by the St Kildans and stored in the dried stomach of a gannet. The living conditions of the St Kildans may also have had an impact with the Islanders sharing their homes with their cattle in winter allowing the dung produced to dry on the floor of the crofts. The introduction of public health nursing methods improved the situation on the Island with no cases of neonatal tetanus reported after 1891 (Woody and Ross 1989).

Aims of Thesis

It has been proposed that *C. botulinum* type C is either carried in the GI tract or is ingested and that an unknown trigger allows the germination of spores and toxin production within the GI tract. To investigate the hypothesis that equine grass sickness is caused by a toxicoinfection with *C. botulinum* type C, the aims of this thesis were:

- To isolate Group III clostridia from the contents of the gastrointestinal tract of horses with equine grass sickness and from control horses and to compare these isolates using phenotypic and genotypic methods.

- To detect BoNT/C producing organisms in the GI tract of horses with and without grass sickness to further investigate the link between a toxicoinfection with *C. botulinum* type C and equine grass sickness independent of the need to culture viable organisms.

- To develop methods for the characterisation of Group III clostridia from a variety of sources, to identify strains that have lost their defining toxin gene and to identify any strain types that are specifically associated with equine grass sickness

- To isolate *C. tetani* and detect the tetanus neurotoxin gene from environmental samples from St. Kilda and from samples of Fulmar oil.

Chapter Two

Materials and Methods

2.1 Isolation and identification of Group III clostridia

Group III clostridia comprise *C. botulinum* types C and D and the closely related *C. novyi* type A.

2.1.1 Bacterial strains

Bacterial strains of Group III organisms (*C. botulinum* type C and D and *C. novyi* type A) *C. botulinum* types A, B and E and of other clostridia were obtained from the Microbial Pathogenicity Research Laboratory (MPRL) collection (Centre for Infectious Diseases, University of Edinburgh College of Medicine and Veterinary Medicine, The Chancellor's Building, 49 Little France Crescent, Edinburgh, UK), the National Culture of Type Strains (NCTC, Central Public Health Laboratory, London, UK), the National Collection of Industrial Bacteria (NCIB, Aberdeen, Scotland) and from the Royal Infirmary of Edinburgh (RIE, Edinburgh) (Table 2.1).

Lab ID	Organism	Source
2509	*C. botulinum* type A	Source unknown
2511	*C. botulinum* type B	NCTC 3732
2512	*C. botulinum* type E	NCTC 8266
2591	*C. sporogenes*	Source unknown
2528	*C. irregularis*	NCIB 11830
2618	*C. butyricum*	Source unknown
2645	*C. sordelli*	NCTC 6801
2651	*C. sphenoides*	NCTC 507
2656	*C. sardiniensis*	Isolate obtained from RIE
2661	*C. perfringens*	NCTC 8257
2713	*C. perfringens*	NCTC 6121
2778	*C. tetani*	NCTC 5413
3209	*C. novyi* type A	RIE 2628
3271	*C. barati*	RIE 2826
3573	*C. tertium*	RIE 4895
3923	*C. botulinum* type D	NCTC 8265
4354	*C. difficile*	RIE 12771
4526	*C. difficile*	Isolate obtained from RIE
4564	*C. botulinum* type C	NCTC 8548

Table 2.1: Bacterial strains used as positive controls for PCR reactions and in Heteroduplex mobility assays.

2.1.2 Samples

Samples used consisted of tonsillar tissue, gastric contents, duodenal tissue and contents, jejunal tissue and contents, colon tissue and contents, rectal tissue and contents, liver and spleen. They were collected from 7 euthanased cases of acute grass sickness. Samples were collected immediately post-mortem from histopathologically confirmed cases. Comparable samples were collected from 5 horses euthanased for non-GI related causes.

2.1.3 Isolation of Group III organisms

Approximately 1g of each specimen was added to 5ml pre-reduced modified Brain-Heart infusion broth. This comprises 37g/L Brain-heart infusion broth (Oxoid, Basingstoke, UK), 10g/L Yeast Extract (Difco), 1g/L L-cysteine-HCl, 10ml/L gentamicin (1mg/ml base, aqueous solution) and cooked meat particles. The samples were incubated anaerobically at 30°C in a DWS Mark III anaerobic work station (Don Whitley Scientific, Shipley, UK). Serial dilutions were plated out after 24h, 2 days and 4 days incubation onto EGS agar comprising 10g/L Trypticase (Becton, Dickson & Company, Sparks, MD), 10g/L Proteose Peptone (Oxoid, Basingstoke, UK), 5g/L Yeast Extract (Difco), 5g/L NaCl, 2.5g/L Sodium succinate and 20g/L Agar (Oxoid, Basingstoke, UK) with the further addition of 0.75g/L L-cysteine HCl, 10mg/L gentamicin, 100ml/L egg yolk emulsion (Oxoid, Basingstoke, UK) and 50ml/L Horse serum (Oxoid, Basingstoke, UK). Plates were checked for the presence of lipase- and lecithinase- positive colonies at 24h intervals. Lipase and lecithinase positive colonies were isolated by sub-culturing onto fresh EGS agar

plates and finally into cooked meat broth to be kept as stock cultures. Colonies positive for lipase only or lecithinase only were also isolated in the same way.

2.1.4 Identification of Group III organisms

Group III organism were identified by their biochemical properties (lecithinase and lipase production), morphology when examined by phase contrast microscopy and Gram stain by light microscopy (Gram positive rods with subterminal oval spores), and gas liquid chromatography profiles (Brown et al, 1996).

2.1.5 Culture of isolates for DNA extraction

Each isolate and reference strain was inoculated into pre-reduced AIM-CMB (Brown et al, 1996) and incubated overnight at 30°C before plating onto Fastidious Anaerobe Agar (Lab M, Bury, UK) supplemented with 10% egg yolk emulsion (Oxoid, Basingstoke, UK). Plates were incubated for up to four days and checked at 24h intervals.

2.1.6 Extraction of DNA for PCR

DNA was extracted from plated colonies using Chelex 100 chelating resin (Bio-Rad, Richmond, CA) according to the method of de Lamballerie et al (1992).

2.1.7 PCR detection of 16S rRNA and clostridial toxin genes

PCR detection of the 16S rRNA gene was used to confirm the presence of bacterial DNA in the samples. The primers used to detect specific toxin genes were chosen from published sequences; the PCR reaction and cycling conditions used were as published for use with those primers. The primer sequences are given in Table 2.2, Reaction conditions are given in Table 2.3 and cycling conditions are given in Table 2.4. All primers were supplied by MWG Biotech. (MWG Biotech AG, Ebersberg, Germany). A Techne TC-412 thermal cycler was used for all PCR reactions. In all PCR reactions the relevant reference strains that were known to contain the particular gene were used as positive controls. A negative control using purified water in place of the template DNA was included in each PCR reaction.

2.1.8 Visualisation of PCR products

PCR products (10 µl) were run on 1% agarose gels (BoNT/C, BoNT/D, novyi α toxin, C2CI and C2CII genes) or 1.5% agarose gels in TAE buffer (40mM Tris and 2mM disodium EDTA, pH8.0) containing ethidium bromide (0.1mg/ml). Products were visualised under ultraviolet light. The readyload 100bp DNA ladder (Invitrogen) was used as a molecular weight marker to size products.

Gene	Primer	Primer sequence	Reference
16s rRNA	16SF	5'-GAACGCGAAGAACCTTAC-3'	(Nubel et al. 1996)
	16SR	5'-TAGCGATTCCGACTTCA-3'	
BoNT/C	Tox384	5'-AAACCTCCTCGAGTTACAAGCCC-3'	(Williamson et al. 1999)
(nested)	Tox625	5'-CTAGACAAGGTAACAACTGGGTTA-3'	
	Tox850*	5'-GAAAATCTACCCTCTCCTACATCA-3'	
	Tox1049*	5'-AATAAGGTCTATAGTTGGACCTCC-3'	
C2CI	C2CIF	5'-AAGGAAGATAAAACAAAAAT-3'	(Fujii et al. 1996)
	C2CIR	5'-CCTAATGATACAAATGAAAA-3'	
C2CII	C2CIIF	5'GCAGAAGTTTCAGGTAGTTTACAAC-3'	(Kimura et al. 1998)
	C2CIIR	5'-CGCATTCTATAACGACCTTCTGGA-3'	
BoNT/D	BoNT/D-F	5'-GTGATCCTGTTAATGACAATG-3'	(Sunagawa et al. 1992)
	BoNT/D-R	5'-TCCTTGCAATGTAAGGGATGC-3'	
A, B & E	BoNT/A-F	5'-AGCTACGGAGGCAGCTATGTT-3'	(Lindstrom et al. 2001)
(multiplex)	BoNT/A-R	5'-CGTATTTGGAAAGCTGAAAAGG-3'	
	BoNT/B-F	5'-CAGGCGAAGTGGAGCGAAAA-3'	
	BoNT/B-R	5'-CTTGCGCCTTTGTTTTCTTG-3'	
	BoNT/E-F	5'-CCAAGATTTTCATCCGCCTA-3'	
	BoNT/E-R	5'-GCTATTGATCCAAAACGGTGA-3'	
C. novyi	Nov-F	5'-GGTGCGATTCAAGAGGCCACA-3'	(Hofmann et al. 1995)
alpha toxin	Nov-R	5'-CGCTCCTAGCAGTCCCGAAAT-3'	
C. perfringens	PA-F	5'-GCTAATGTTACTGCCGTTGA-3'	Songer & Bueschel,
alpha toxin	PA-R	5'-CCTTCTGATACATCGTGTAAG-3'	1999
C. perfringens	PB-F	5'-GCGAATATGCTGAATCATCTA-3'	Songer & Bueschel,
beta toxin	PB-R	5'-GCAGGAACATTACTATATCTTC-3'	1999
C. perfringens	PE-F	5'-GGAGATGGTTGGATATTAGG-3'	Songer & Bueschel,
enterotoxin	PE-R	5'-GGACCAGCAGTTGTAGATA-3'	1999

Table 2.2: Primer sequences for amplification of target genes.

* denoted the reverse primers for the nested PCR.

Gene	RedTaq Buffer	$MgCl_2$	dNTP	Primer	Red Taq
16s rRNA C2CI, C2CII, BoNT/D *C. novyi* alpha toxin	1X (50mM KCl, 10mM Tris-HCl)	1.5mM	12.5mM	10pmol.µl	2.5U
BoNT/C	1X (50mM KCl, 10mM Tris-HCl)	3.75mM	0.2mM	1µM	1.25U
A, B &E multiplex	1X (50mM KCl, 10mM Tris-HCl)	4.8mM	0.22mM	0.3µM	2U
C. perfringens alpha, beta and enterotoxin	1X (50mM KCl, 10mM Tris-HCl)	2.0mM	0.12mM	10pmol/µl	5U

Table 2.3: Reaction conditions for PCR using primer pairs detailed in Table 2.2. All reactions were carried out in a total volume of 100µl with 5µl of template DNA. PCR buffer and Taq polymerase were supplied by Sigma (Poole, Dorset).

Gene	Initial denaturation	Cycles	Cycling conditions	Final extension
16s rRNA	5 min at 94°C	35	1 min at 94°C	5 min at 72°C
			1 min at 63°C	
			1 min at 72°C	
BoNT/C	5 min at 80°C	30	1 min at 95°C	10 min at 72°C
			1 min at 55°C	
			1 min at 72°C	
C2CI	3 min at 94°C	35	45s at 94°C	5 min at 72°C
			2 min at 45°C	
			1 min at 72°C	
C2CII	3 min at 94°C	40	45s at 94°C	5 min at 72°C
			1 min at 53°C	
			3 min at 72°C	
BoNT/D	10 min at 95°C	25	1 min at 94°C	3 min at 72°C
			1 min at 55°C	
			1 min at 72°C	
A, B & E	3 min at 94°C	28	30s at 95°C	3 min at 72°C
multiplex			25s at 60°C	
			1 min 25s at 72°C	
C. novyi alpha	3 min at 94°C	30	1 min at 95°C	5 min at 72°C
toxin			1 min at 48°C	
			1 min at 72°C	
C. perfringens	5 min at 94°C	35	1 min at 94°C	5 min at 72°C
alpha, beta and			1 min at 50°C	
enterotoxins			1 min at 72°C	

Table 2.4: Cycling conditions for PCR reactions using primer pairs detailed in Table 2.2.

2.1.9 Immunomagnetic separation (IMS) of Group III clostridia

Coating of magnetic beads

Aliquots (33µl) of Dynal magnetic beads coated in anti-rabbit immunoglobulin (Invitrogen) were transferred to sterile 1.5ml tubes and washed three times in 1ml phosphate buffered saline, pH 7.2 containing 0.1% bovine serum albumen (PBS-BSA) using the magnetic particle collector. Serum obtained from rabbits inoculated with cell-surface extracts of non-toxigenic *C. botulinum* type C (MPRL 2510) was diluted 1 in 100, 1 in 1000 and 1 in 10000 in PBS-BSA. The beads were re-suspended in 1ml of the serum dilutions and incubated overnight at 4˚C on a rotating mixer.

Beads were washed four times in PBS and re-suspended in a 1 in 1000 dilution of anti-rabbit IgG-FITC conjugate in PBS. Fluorescence microscopy was used to determine which serum dilution gave adequate coating of the beads.

On the basis of this, a 1 in 100 serum dilution was used for all further IMS work.

Sample preparation for IMS

Pre-coated beads stored in PBS-BSA with sodium azide were transferred to an anaerobic workstation and washed twice in pre-reduced PBS-BSA. All wash steps were carried out in a volume of 1ml.

Pure culture of *C. botulinum* type C

An overnight culture of *C. botulinum* type C (MPRL 4564) was diluted to give a concentration of $1x10^6$ cells/ml based on total cell counts using a Thoma cell counting chamber.

Mixed bacterial culture

Overnight broth cultures of *C botulinum* type C (MPRL 4564), *C. sporogenes* (MPRL 2591) *C. sordelli* (MPRL 2645) and *C. perfringens* (MPRL 2661) were each diluted to a concentration of $1x10^6$ cells/ml based on total cell counts and equal volumes combined. Duplicate tubes were also set up with 0.1% Tween-20 added to the nutrient broth used in the washing stages to reduce non-specific binding of bacteria to the beads.

Spiked faecal enrichment culture

Approximately 1g healthy horse faeces was added to 5ml pre-reduced AIM-CMB and transferred to an anaerobic workstation. An overnight culture of *C. botulinum* type C was diluted to a concentration of $1x10^6$ cells/ml and 0.5ml added to the faecal enrichment culture. Following 48h incubation, the culture supernate was diluted 1 in 100, 1 in 1000 and 1 in 10000.

Recovery of *C. botulinum* type C

The beads were initially incubated with 1ml of the bacterial suspension on a rotating mixer for 15min, 30min, 45min or 60min. On the basis of the initial experiment using a pure culture of *C. botulinum* type C, subsequent samples were incubated with

the beads for 30 min. Following incubation, beads were washed four times in pre-reduced nutrient broth with the addition of 0.1% Tween-20 and re-suspended in 1ml nutrient broth from which dilutions of 1 in 10 and 1 in 100 were prepared and 20µl aliquots used to inoculate FAA plates for viable cell counts following 24h incubation at 30˚C. The starter culture was also plated out at the same dilutions for viable cell counts in order to determine the percentage recovery.

Recovery of Group III clostridia from enrichment cultures

Enrichment broth cultures of equine gastrointestinal contents following 48h incubation were diluted 1 in 100, 1 in 1000 and 1 in 10000 and subjected to the immunomagnetic separation procedure as previously described. The resulting bead suspension was diluted to 1 in 100, 1 in 1000 and 1 in 10000 and used to inoculate EGS plates. Plates were checked after 24h incubation at 30˚C and representative colonies were sub-cultured to obtain pure cultures and stored in cooked meat broths to be used as stock cultures.

2.1.10. Direct detection of BoNT/C by ELISA

Processing of samples for detection of BoNT/C

For the detection of BoNT/C, isolates obtained from the enrichment cultures of equine gastrointestinal contents were inoculated into fresh AIM-CMB and incubated

anaerobically at 30˚C for 4 days. The culture supernate was removed and centrifuged at 10000g for 10 min and the supernate collected and stored at -20˚C.

ELISA to detect BoNT/C

A sandwich ELISA for the detection of BoNT/C had been developed by CAMR, Porton Down. A polyvalent guinea pig antiserum (supplied by CAMR) raised against the purified neurotoxin was used as a capture antibody. Nunc Immuno™ Polysorp™ ELISA strips (Fisher Scientific UK, Loughborough, Leicestershire, UK) were coated (100µl per well) overnight at 4°C with 5-10µg/ml of the antiserum diluted in PBS pH 7.4. Plates were washed four times with PBS containing 0.1% Tween 20 (PBS-T).

Plates were blocked with PBS containing 3% teleostean gelatine (PBS-TG; Sigma; 200µl per well) for 2h at 37°C in a shallow water bath and then washed four times in PBS-T and stored at -20°C until use.

Culture supernates were assayed in duplicate at two dilutions (undiluted and 1 in 4 for direct detection and undiluted and 1 in 10 for enrichment cultures). Samples were diluted in PBS-TG and 100µl added to each well. The plates were incubated at 37°C for 90min, with shaking, and then washed four times with PBS-T.

To detect the BoNT/C, the anti-BoNT/C guinea pig IgG (supplied by CAMR), conjugated to horseradish peroxidase (HRP) was used at a dilution of 1 in 300 in PBS-TG (100µl per well). Plates were incubated for 90 min at 37°C and then washed four times in PBS-T.

The substrate (3, 3`, 5, 5`-tetramethyl-benzidine dihydrochloride tablets, Sigma) dissolved in phosphate-citrate buffer pH 5.0 with 2µl 30% H_2O_2 per 100ml, was

added to the plate (100µl per well). The reaction was allowed to develop at room temperature for 30min or until the background became detectable. The reaction was stopped by the addition of 2M H_2SO_4 (50µl per well). The absorbance was measured at 450nm, reference at 620nm, in an Anthos plate reader.

Controls

Purified BoNT/C was diluted in PBS-TG to 200ng/ml, 50ng/ml, 12.5ng/ml, 3.12ng.ml and 0.8ng/ml and was included as a standard to enable comparison between plates and quantification of levels of toxin detected.

Columns 1, 6, 7 and 12 were not coated, receiving only PBS-G (100µl/well) during the coating stage. Each sample was therefore added to two coated wells and one uncoated well. The uncoated well was used as a control for non-specific binding of the sample to the plate. Two rows of wells received no samples, only PBS-G (100µg/well) as a negative control.

Calculation of ELISA results

The mean OD for the negative control was subtracted from the mean OD for the sample in the coated wells and from the OD of the sample in the uncoated well. The OD for the uncoated well (minus the negative control) was then subtracted from the mean sample OD. This value was used to calculate the amount of BoNT/C present in the samples, using the standard BoNT/C curve and correcting for dilution factors. Samples were considered positive if the final OD value was equal to or greater than that for the 0.8ng/ml BoNT/C standard.

2.1.11 Detection of the BoNT/C gene in enrichment cultures by PCR

Processing of samples for DNA extraction

Approximately 1g of each of the gastrointestinal content and tissue samples was added to 5ml pre-reduced AIM-CMB (Brown et al, 1996) and transferred to an anaerobic cabinet. After 24h and 5 days incubation at 30˚C, a 1ml aliquot of culture supernate was removed, centrifuged at 10000g for 5 min and the pellet stored at -20°C.

Extraction of DNA for PCR

The pellet was resuspended in 597µl Tris-EDTA buffer pH8.0 (TE buffer; 10mM Tris and 1mM EDTA) with 3µl Proteinase K ($20mg.ml^{-1}$) and lysed using a Mini Bead Beater-8 (BioSpec Products Ltd) for 3 min with 0.1mm silica/zirconium beads (BioSpec Products Ltd). The supernate was removed to a fresh tube with 100µl 5M NaCl and 80µl CTAB solution was added to precipitate humic acids and incubated at 37°C for 10min. An equal volume of chloroform:isoamyl alcohol mixture (Sigma) was added and shaken to form an emulsion before centrifugation at 10000g for 5min. The clear fraction was removed to a fresh tube and an equal volume of phenol:chloroform:isoamyl alcohol mixture was added (Sigma), shaken to form an emulsion and centrifuged at 10000g for 5 min. The aqueous supernate was removed and the DNA precipitated by the addition of 0.6 volumes isopropanol. The tube was gently shaken and centrifuged at 10000g for 1 min to pellet the DNA. The supernatant was removed and the pellet washed with 70% ethanol and centrifuged at 10000g for 5 min. The pellet was dried in a 37°C incubator for 10 min and re-suspended in 100µl TE buffer overnight at 4°C.

Detection of the Group III clostridial genes by PCR

PCR detection of the genes for the BoNT/C, BoNT/D, C2 toxin and *C. novyi* alpha toxin was carried out using the primer sets and reaction conditions detailed in Table 2.2, Table 2.3 and Table 2.4.

2.1.12 Sequence analysis of BoNT/C PCR products

Preparation of samples for sequencing.

PCR products generated using the Tox384/1049 primer pair were separated by agarose gel electrophoresis and the bands excised using sterile disposable scalpels over an ultraviolet light source. The PCR product was extracted from the gel using the MinElute Gel Extraction kit (Qiagen) according to the manufacturer's protocol and used as the DNA template for the PCR reaction using the Tox625/Tox850 primer pair. PCR products were separated by agarose gel electrophoresis and the PCR products extracted from the gel using the MinElute Gel Extraction kit (Qiagen). The absorbance at A280/A260 was measured to quantify the amount of DNA present and the PCR products were sent to the Moredun Institute for sequencing (Functional Genomics Unit, Moredun Research Institute, Edinburgh, UK).

Analysis of sequence data

Sequence data was converted into FASTA format using the Chromas 2 program and entered into a BLAST search for comparison with published gene sequences. All sequences that were found to match, or partially match, the BoNT/C sequence were aligned using Multalin (Corpet, 1988).

2.1.13 Detection of BoNT/C gene products by Southern blotting

After separation by agarose gel electrophoresis, PCR products were denatured by incubation in denaturation buffer (1.5M NaCl; 0.5M NaOH) for 30min at room temperature with gentle shaking followed by incubation in neutralisation buffer (1M Tris·Cl; 1.5M NaCl, pH 7.4) for 30 min at room temperature with gentle shaking. PCR products were then transferred to positively charged nylon membrane (0.45µm pore size) overnight in 10X sodium citrate buffer (SSC; 1.5M NaCl, 0.15M sodium citrate, pH 7.0). The gel was discarded and the PCR products cross linked to the nylon membrane by exposure to UV light for 30s. The nylon membrane was then incubated in Pre-hybridisation buffer containing 5xSSC; 0.1% N-laurolysarcosine (w/v), 0.02% SDS (w/v) and 1% Blocking solution. DIG labelled probes (MWG Biotech) were denatured at 95°C for 10 min and diluted 1:10000 in Pre-hybridisation buffer and incubated with the membrane overnight at 60°C. All of the following steps were carried out at room temperature with gentle shaking. The nylon membrane was washed twice in SSC (2x) with 0.1%SDS, then washed twice in 0.5xSSC with 0.1%SDS and blocked for 30min with Blocking solution (Roche). Following blocking, the nylon membrane was incubated in a 1:5000 dilution of anti-DIG antibody in blocking solution and washed twice in Washing buffer (Roche). The nylon membrane was then incubated in Detection buffer for 5min before developing in freshly prepared substrate solution in the dark. When the required colour intensity was achieved the reaction was stopped by rinsing in double distilled water.

Screening of BoNT/C PCR products by slot blotting

Slot blotting of PCR products to detect the BoNT/C gene from enrichment cultures was carried out using the Bio-Dot Microfiltration apparatus (Bio-Rad). Nylon membrane (0.45µm pore size, Roche) was soaked in SSC for 10 min and the slot blotting apparatus assembled according to the manufacturer's guidelines. Wells were rinsed with 100µl distilled water and 75µl PCR products applied to the membrane and pulled through using a vacuum pump. The membrane was removed and placed, face up on a sheet of filter paper soaked in 10%SDS solution for 5 min, then placed on filter paper soaked in denaturation buffer for 5 min, neutralisation buffer for 5 min and finally SSC (2x) for 5 min. The membrane was exposed to ultraviolet light for 30s in a transilluminater and then incubated in Pre-hybridisation buffer containing 5xSSC; 0.1% N-laurolysarcosine (w/v). 0.02% SDS (w/v) and 1% Blocking solution overnight at 60°C. DIG labelled probes (MWG Biotech) were denatured at 95°C for 10 min and diluted 1:10000 in Pre-hybridisation buffer and incubated with the membrane overnight at 60°C. All of the following steps were carried out at room temperature with gentle shaking. The nylon membrane was washed twice in SSC (2x) with 0.1%SDS, washed twice in 0.5xSSC with 0.1%SDS and blocked for 30min with blocking solution. Following blocking, the nylon membrane was incubated in a 1:5000 dilution of anti-DIG antibody in blocking solution and washed twice in Washing buffer. The nylon membrane was then incubated in Detection buffer for 5min before developing in freshly prepared substrate solution in the dark. When the required colour intensity was achieved the reaction was stopped by rinsing in double distilled water.

2.2 Detection of Group III toxin genes in C. novyi type A, C. botulinum type C and C. botulinum type D

2.2.1 Bacterial strains

Bacterial strains of Group III organisms were obtained from the National Culture of Type Strains (NCTC, Central Public Health Laboratory, London, UK), the Glaxo collection and the MPRL collection, the Public Health Laboratory Service (PHLS), the Anaerobe Reference Unit (ARU; Cardiff) (Table 2.5).

2.2.2 Culture of strains for DNA extraction

Bacterial strains from the laboratory freeze-dried culture collection were inoculated into pre-reduced AIM-CMB and transferred to an anaerobic workstation. Following overnight incubation at 30˚C, strains were plated onto Fastidious Anaerobe Agar (Lab M, Bury, UK) supplemented with 10% egg yolk emulsion (Oxoid, Basingstoke, UK). Plates were checked after 24h and 48h incubation at 30˚C.

2.2.3 Extraction of DNA for PCR

DNA was extracted from plated colonies using Chelex 100 chelating resin (Bio-Rad, Richmond, CA) according to the method of de Lamballerie et al (1992).

Lab No.	Organism	Source
141	*C. novyi* type A-like	Horse ileum (subacute EGS)
465	*C. novyi* type A	Wound site (common bile duct drain)
2306	*C. novyi* type A	Source not known
2307	*C. novyi* type A	Source not known
2510	*C. botulinum* type C	NCTC 3732
2530	*C. novyi* type A	NCTC 538
2531	*C. novyi* type A	Glaxo collection
2533	*C. novyi* type A	NCTC 6735
2534	*C. novyi* type A	Source not known
2535	*C. novyi* type A	Source not known
2536	*C. novyi* type A	Source not known
3209	*C. novyi* type A	PHLS 2680
3341	*C. novyi* type A	Soil (St. Kilda)
3923	*C. botulinum* type D	NCTC 8265
4540	*C. novyi* type A	Injection site (Glasgow)
4541	*C. novyi* type A	Injection site (Glasgow)
4545	*C. novyi* type A	Crush injury wound swab
4547	*C. novyi* type A	Injection site (PHLS Cardiff)
4548	*C. novyi* type A	Injection site (PHLS Cardiff)
4549	*C. novyi* type A	Horse ileum (AGS)
4550	*C. novyi* type A	Horse ileum (AGS)
4551	*C. novyi* type A	Horse ileum (AGS)
4552	*C. novyi* type A	Hare with dysautonomia, small intestine
4553	*C. novyi* type A	Hare with dysautonomia, small intestine
4554	*C. novyi* type A	Hare with dysautonomia, small intestine
4556	*C. novyi* type A	Horse faeces, CGS
4557	*C. novyi* type A	Horse faeces, recovered CGS
4558	*C. novyi* type A	Horse faeces, contact EGS
4559	*C. novyi* type A	Horse faeces, contact EGS
4561	*C. novyi* type A	Horse ileum, AGS
4562	*C. novyi* type A	Horse ileum, CGS
4563	*C. novyi* type A	Horse faeces, CGS
4564	*C. botulinum* type C	NCTC 8548
4565	*C. botulinum* type C	NCTC 10914
4566	*C. novyi* type A	Injection site (Glasgow)
4567	*C. novyi* type A	Injection site (Glasgow)
4568	*C. novyi* type A	NCTC 6738
4569	*C. novyi* type A	Injection site (Dublin)
4570	*C. novyi* type A	ARU collection
4571	*C. novyi* type A	NCTC 538
4572	*C. novyi* type A	Injection site (Bournemouth)
4573	*C. novyi* type A	Injection site (Glasgow)
4574	*C. novyi* type A	Injection site (Glasgow)
4575	*C. novyi* type A	Injection site (Stockton)

Table 2.5: Group III reference strains used. All isolates are from human sources unless otherwise stated.

2.2.4 PCR detection of Group III clostridial toxin genes

PCR for the detection of the BoNT/C, BoNT/D, C2CI, C2CII and novyi alpha toxin genes was carried out using the primers detailed in Table 2.2. PCR reaction and cycling conditions are given in Table 2.3 and Table 2.4.

2.2.5 Sequence analysis of the C2CI and 16s rRNA PCR products

Extraction and sequencing of the C2CI and 16s rRNA PCR products was performed as described for the sequencing of the BoNT/C gene PCR products (Chapter 2.1.12)

2.2.6 Heteroduplex mobility assay

PCR using the 16SF/16SR primer pair was performed as previously described and 5µl PCR product from each of the clostridial strains (Table 2.1) or the Group III-type organisms (Table 2.5) were combined with 5µl PCR product from *C. botulinum* type C (MPRL 4564) in denaturing buffer (1M NaCl, 100mM Tris-HCl, 20mM disodium EDTA, pH 8.0) and incubated at 95˚C for 10 min. Products were separated on a slab gel (12.5% acrylamide in 5X TBE). Gels were stained with 1mg/ml ethidium bromide in 0.6X TBE and visualised using ultraviolet light.

2.3 Detection of botulinum neurotoxins and Group III clostridial toxins in seabirds

2.3.1 Direct detection of BoNT/C by ELISA

Approximately 1g/1ml of each sample of the gastrointestinal contents of seabirds was added to 5ml pre-reduced AIM-CMB and transferred to an anaerobic workstation. Following incubation at 30˚C for 24h and 4 days, a 1ml aliquot of culture supernate was removed and centrifuged at 10000g for 10 min and the supernate stored at -20˚C for toxin detection. The pellet was also stored at -20 ˚C for DNA extraction.

Serum samples were centrifuged at 13000rpm for 10 min and the supernatant used directly for the detection of BoNT/C by ELISA (Chapter 2.1.10).

2.3.2 Detection of toxin genes by PCR

Extraction of DNA for PCR

DNA was extracted from the pellets of enrichment cultures of the gastrointestinal contents of seabirds (Table 2.7) using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol. PCR primer details and reaction conditions for the detection of the BoNT/A, BoNT/B, BoNT/C, BoNT/D and BoNT/E genes and the novyi alpha toxin gene and C2CI and C2CII genes are given in Table 2.2, Table 2.3 and Table 2.4.

Sample	Source
B303427/1	GI contents (herring gull)
B303427/2	GI contents (herring gull)
B303467	GI contents (gannet)
B303515/1	GI contents (herring gull)
B303515/2	GI contents (herring gull)
B303534/2	GI contents (herring gull)
B303853/1	Liver and Intestine (herring gull)
B303853/2	Liver and Intestine (herring gull)
B303853/3	Liver and Intestine (herring gull)
B303853/4	Liver and Intestine (herring gull)
B303855/1	GI contents (herring gull)
B303855/2	GI contents (herring gull)
B303855/3	GI contents (herring gull)
B303855/4	GI contents (herring gull)
B303855/5	GI contents (herring gull)
B303855/6	GI contents (cormorant)
B304150	Intestine from 5 herring gulls (mixed sample)
B301155a	Leeches recovered from herring gulls
B301155b	Leeches recovered from herring gulls
B301155c	Leeches recovered from herring gulls

Table 2.7: Seabird samples used. All samples provided by the Scottish Agricultural College (Ayr, Scotland)

2.4 Detection of C. tetani

2.4.1 Isolation of *C. tetani*

Four samples of soil and environmental material were collected from St. Kilda. Approximately 1g of each sample was added to 5ml pre-reduced AIM-CMB and transferred to an anaerobic workstation. The resulting broth cultures were used to inoculate Colombia Blood Agar plates containing Colombia agar base (Oxoid, Basingstoke, UK) supplemented with 5% horse blood (Oxoid, Basingstoke, UK) after 0h, 24h and 48h incubation at 37°C. Plates were incubated at 37 °C and were checked at 24h intervals for the presence of fine swarming growth. Any finely swarming colonies were subcultured onto CBA containing 4% agar and incubated at 37 °C. Plates were checked for the presence of faintly haemolytic colonies. After 24h and 4 day incubation 1ml culture supernate was removed, centrifuged at 10000g for 10 minutes and the pellet stored at -20 °C for DNA extraction.

C. tetani was presumptively identified by morphology when examined by phase contrast microscopy and Gram stain by light microscopy (Gram positive rods with circular terminal or sub-terminal spores).

Forty samples of "Fulmar oil" (stomach contents of nestlings spat out as a form of defence) were collected from nesting birds in the Firth of Forth and stored at -20 °C. Approximately 1ml of each sample was added to pre-reduced AIM-CMB for the isolation of *C. tetani* as above. Oil samples were also plated directly onto CBA plates and incubated at 37 °C in an anaerobic workstation.

2.4.2 Detection of the Tetanus neurotoxin by PCR

DNA was extracted from the pellets of enrichment cultures of soil and Fulmar oil samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol.

All PCR reaction were carried out in 100µl reaction buffer (Sigma) containing 3.75mM $MgCl_2$, 50nM of each primer, 200µM dNTP, 5µl DNA template and 1.25U RedTaq (Sigma). The primers were taken from a published sequence (Halpern et al, 1990). PCR cycling conditions are given in Table 2.8.

PCR products were separated on a 1.5% agarose gel in TAE buffer (40mM Tris and 2mM disodium EDTA, pH8.0) containing ethidium bromide (0.1mg/ml). Products were visualised under ultraviolet light. The readyload 100bp DNA ladder (Invitrogen) was used as a molecular weight marker to size products.

Primer sequences

Tet-F 5'-CGCGTCGACTCAACACCAATTCCATTTTCTTATTC-3'

Tet-R 5'-GCGCTGCAGTCATGAACATATCAATCTGTTTAATC-3'

Gene	Initial denaturation	Cycles	Cycling conditions	Final extension
TetC	5 min at 94°C	25	2 min at 94°C 3min at 30°C 10min at 67°C	5 min at 67°C

Table2.8: PCR cycling conditions for the detection of the gene for Fragment C of the Tetanus neurotoxin.

Chapter Three

Isolation and Characterisation of Group III organisms from equine gastrointestinal contents

The aetiology of grass sickness remains unknown though serological data supports the current hypothesis that grass sickness is caused by an intoxification with *C. botulinum* type C (Hunter and Poxton 2001; MacCarthy et al. 2004).

In order to further investigate the possible relationship between Equine Grass sickness and *C. botulinum* type C the following work was carried out.

3.1 Results

3.1.1 Isolation of Group III organisms

All culture work was carried out in conjunction with Bob Brown and Fraser Pike (MPRL). A total of 464 isolates were collected from the enrichment culture of tissue and gastrointestinal contents of horses with histopathologically confirmed cases of grass sickness, and from faecal samples taken from co-grazers (Figure 3.1). From the control horses used in the study 128 isolates were obtained. All isolates were Gram positive rods as seen under a light microscope and produced lecithinase, lipase or both on EYA. The organisms were sub-cultured on EYA plates until pure cultures were obtained. Seven of the isolates obtained from horses with EGS produced proprionic and butyric volatile fatty acids (VFA) along with varying amounts of acetic and valeric VFA (Table3.1). These GLC profiles are characteristic of the Group III clostridia.

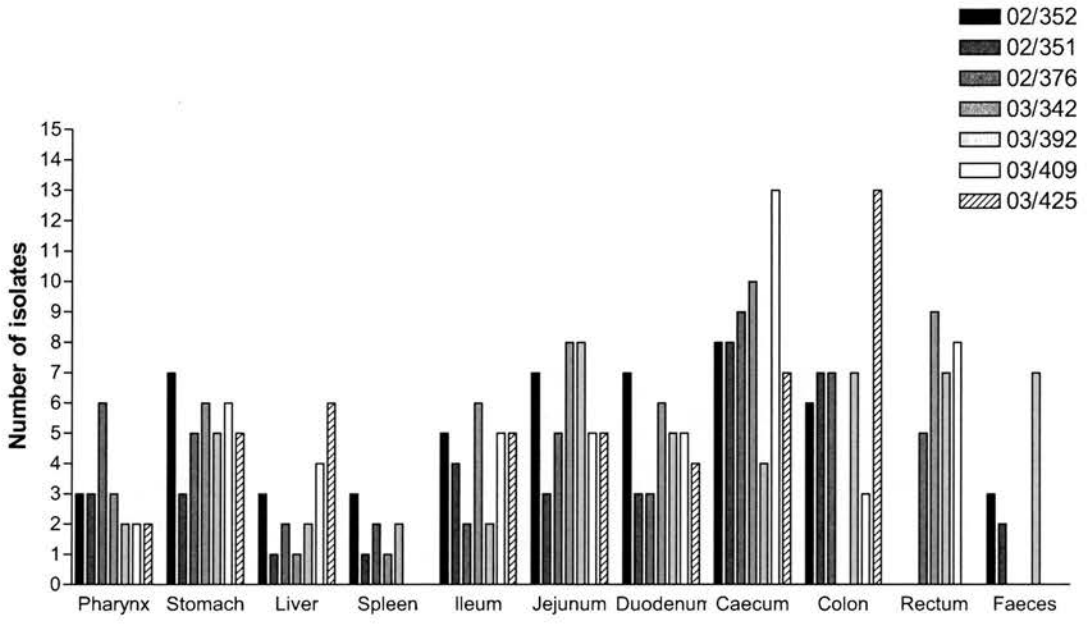

Figure 3.1: Source of isolates obtained from horses with EGS

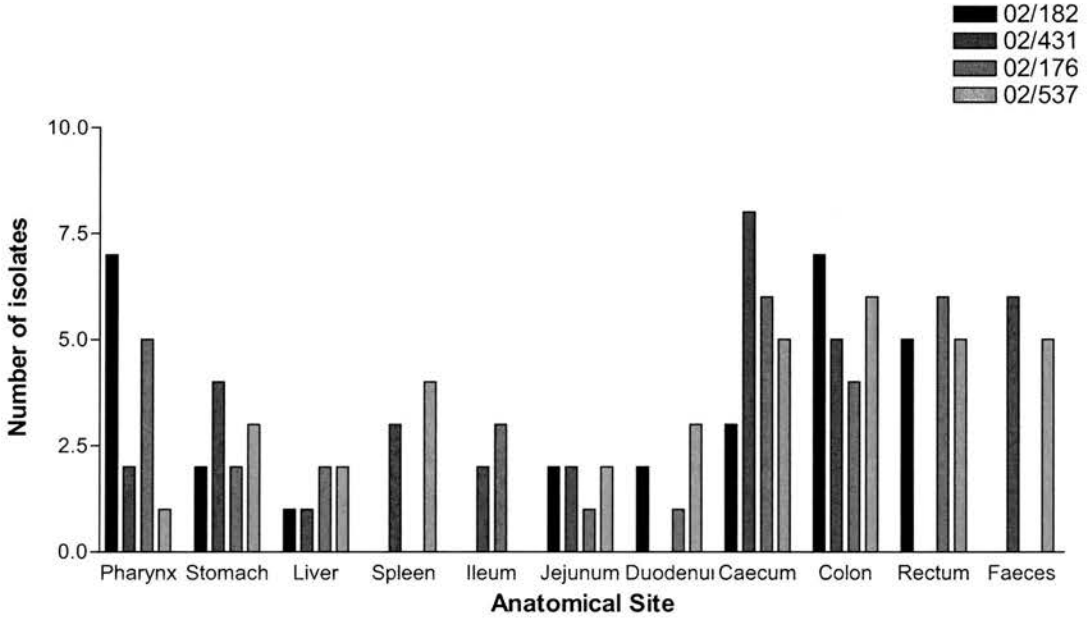

Figure 3.2: Source of isolates obtained from control horses

Isolate	Source of isolate	Sample type	Volatile fatty acids
RBGS 553	Horse 02/351	Liver	APb
RBGS 116	Horse 02/352	Faeces	apB(iv)
RBGS 559	Horse 02/352	Faeces (co-grazer)	APB
RBGS 486	Horse 02/376	Pharynx	aPB(v)
RBGS 488	Horse 02/376	Pharynx	PBv(a)
RBGS 637	Horse 03/342	Rectal contents	PBv(a)
RBGS 887	Horse 02/392	Faeces (co-grazer)	Apb

Table 3.1: GLC profiles of isolates identified as Group III clostridia and description of the animal and sample from which they were obtained. P = proprionic acid, B = butyric acid, A = acetic acid, V = valeric acid. Letters in upper case represent levels of fatty acids above 10µmoles/ml, letters in lower case represent levels of fatty acids between 1 – 10 µmoles/ml, and letters in brackets represent levels of fatty acids between 0.2 – 1 µmoles/ml.

3.1.2 Characterisation of isolates

A random sample of 64 isolates were investigated for the presence of clostridial toxin genes by PCR in conjunction with Allison Wroe (MPRL), 29 had been isolated from horses with confirmed grass sickness, 10 from faeces of co-grazers of cases with grass sickness and 25 from the control animals.

Grass Sickness isolates

Of the isolates obtained from grass sickness samples, 16 were found to contain no toxin genes by PCR. Phenotypic characterisation of these isolates shows that 9 of the isolates resemble *C. sporogenes* and were found throughout the GI tract, 5 of the isolates were novyi-like and these were found only in the large intestine. In addition one isolate was found to resemble *C. sordelli* and one *C. septicum*.

None of the isolates tested were found to contain the A, B, C or D botulinum neurotoxins or the *C. novyi* α toxin genes by PCR. Toxigenic isolates were found in a variety of samples types (Table 3.2). Six of the isolates appeared to have atypical toxin profiles for the phenotype and one isolate (544) appeared to have an unusual combination of toxin genes carrying genes typical of both the Group III and Group II clostridia.

Isolate #	Source	Phenotype	C2CI	C2CII	PCR results BoNT/E	*C. perfringens* α toxin
115	Faeces (CGS)	sporogenes-like	+	-	NCR	NCR
266	Ileum	perfringens-like	-	-	NCR	+
340	Spleen	perfringens-like	+	-	NCR	NCR
423	Colon	perfringens-like	-	+	NCR	NCR
459	Pharynx	perfringens-like	-	-	NCR	+
498	Colon	perfringens-like	-	-	NCR	+
528	Caecum	novyi-like	-	-	-	+
544	Colon	novyi-like	-	+	+	-
548	Caecum	perfringens-like	-	-	-	+
624	Duodenum	perfringens-like	-	-	-	+
633	Duodenum	sporogenes-like	-	-	-	+
659	Stomach	novyi-like	-	-	-	+
722	Rectum	novyi-like	+	-	NCR	NCR

Table 3.2 Phenotype and toxin gene profile of toxigenic isolates (n=13) from grass sickness cases.
NCR – no consistent result obtained for this isolate.

Co-grazer isolates (faecal isolates only)

No isolates were found to contain the genes for the A, B, C, D or E botulinum neurotoxins or the *C. novyi* α toxin. The majority of the isolates examined resemble *C. novyi*. One isolate resembled a Group I botulinum-like organism, one *C. sordelli* and one *C. sporogenes*. Two isolates were found to carry genes for one or more component of the C2 toxin. Isolate 147 contains only the CI component and isolate 895 contains both components of the C2 toxin gene.

Control animal isolates

Twenty-five of the isolates studied were from control animals. Of these 17 were found to contain no toxin genes by PCR. The majority of the isolates (12) were found to be novyi-like by phenotypic characterisation with 8 resembling *C. sporogenes*, 3 resembling *C. perfringens* and 1 resembling *C. sordelli*. No isolates were found to contain the A, B, C, D or E botulinum neurotoxins or the *C. novyi* α toxin genes. Toxigenic isolates were found throughout the GI tract (Table 3.3). Again atypical isolates were found; isolate 411 contains genes characteristic of the Group III clostridia but phenotypically resembles *C. sporogenes* and isolate 725 contains genes typical of both *C. perfringens* and the Group III clostridia.

Isolate #	Source	Phenotype	PCR results C2CI	C2CII	*C. perfringens* α toxin
411	Jejunum	sporogenes-like	+	-	NCR
464	Pharynx	UD	-	+	NCR
468	Pharynx	perfringens-like	-	-	+
607	Spleen	perfringens-like	+	-	NCR
675	Caecum	perfringens-like	-	-	+
725	Rectum	sporogenes-like	-	+	+
748	Caecum	novyi-like	+	-	NCR
786	Colon	novyi-like	+	-	NCR

Table 3.3 Phenotype and toxin gene profile of toxigenic isolates from control animals.

NCR – no consistent result obtained for this isolate

UD – phenotype could not be determined on the basis of biochemical properties or GLC profile.

3.1.3. Immunomagnetic separation of Group III clostridia

An immunomagnetic separation technique for the isolation of Group III clostridia was developed. Magnetic beads coated in anti-rabbit antibodies (Dynatech) were coated overnight in the pooled serum from rabbits inoculated with whole killed cells of a non-toxin producing strain of *C. botulinum* (MPRL 4565) on a rotating mixer at 4°C. Coating was verified using fluorescence microscopy with FITC labelled anti rabbit immunoglobulin.

Coated beads were incubated anaerobically with a pure culture of *C. botulinum* in pre-reduced AIM/CMB for 15, 30, 45 and 60 mins to determine the optimum time for binding of bacterial cells to the coated beads. The resulting suspension was used to inoculate both AIM/CMB to check for toxin production and FAA plates to determine the percentage recovery.

The optimum recovery of cells was 33.5% after 30 mins incubation with the magnetic beads. An incubation time of 10 mins gave 23% recovery; 45 mins incubation gave a recovery of 13.5% and 60 mins incubation gave a recovery of only 8.6%.

In mixed clostridial cultures (*C. botulinum* type C, *C. sporogenes, C. sordelli* and *C. perfringens*) recovery of *C. botulinum* was poor (16.5%) and hard to distinguish from remaining *C. sporogenes*. The addition of TWEEN-20 gave a 45% recovery of *C. botulinum* though large numbers of *C. perfringens* remained.

As this technique gave a 57.5% recovery of *C. botulinum* type C from spiked healthy horse faeces it was applied to two samples from the Dubai study; the ileal contents from horse 03/342 and from the large colon contents of 'Horse G'. A representative sample of the colony types produced were further purified and characterised by phenotypic methods and direct detection of the BoNT/C as well as PCR detection of toxin genes (Table 3.4). All of the isolates characterised phenotypically appear to have atypical toxin profiles for their phenotype with none resembling Group III clostridia. Two of the isolates that tested positive for the BoNT/C gene by PCR gave bands of an atypical size of approximately 600bp rather than the expected 460bp based upon the published sequence, but were found to produce detectable levels of the BoNT/C by ELISA-based toxin assay.

Isolate	Source	Phenoytpe	Toxin (ng/ml)	PCR BoNT/C	C2CI	C2CII
1	Ileum	*C. bifermentans* - like	14.4	+*	-	-
2	Ileum	*C. sordelli* – like	8.4	+*	+	+*
3	Ileum	*C. perfringens* – like	15	+	+	-
4	Ileum	UD	8	-	+	+
5	Ileum	UD	<0.8	-	-	+
6	Colon	*C. perfringens* – like	14.8	+	+*	+
7	Colon	UD	<0.8	-	-	-
8	Colon	*C. sordelli* - like	<0.8	+*	-	+*

Table 3.4. Phenotype, toxin gene profile and toxin production of isolates obtained by immunomagnetic separation
* - PCR product not expected size

UD – phenotype could not be determined on the basis of biochemical properties or GLC profile.

3.1.4. Investigation of Group III clostridial genes in enrichment cultures from horses with Grass Sickness by PCR

PCR detection of the BoNT/D gene

PCR with the BoNT/D primers produced a 497bp product from DNA of *C. botulinum* type D demonstrating the presence of the BoNT/D gene. None of the DNA extracts from enrichment cultures were found to contain the gene.

PCR detection of the C2 toxin gene

PCR with the C2CI primers amplified a 310bp product in the *C. botulinum* type C strain (MPRL 1464) demonstrating the presence of the component I gene of the C2 toxin. Additional bands of varying sizes between 500 and 900 bp were amplified from all of the samples with many also producing a 310bp product (Fig3.3).

PCR with the C2CII primers amplified a 1093bp product with DNA from the *C. botulinum* type C strain (MPRL 4564) demonstrating the presence of the component II gene of the C2 toxin. None of the gut enrichment cultures demonstrated the presence of this gene despite 2 rounds of amplification.

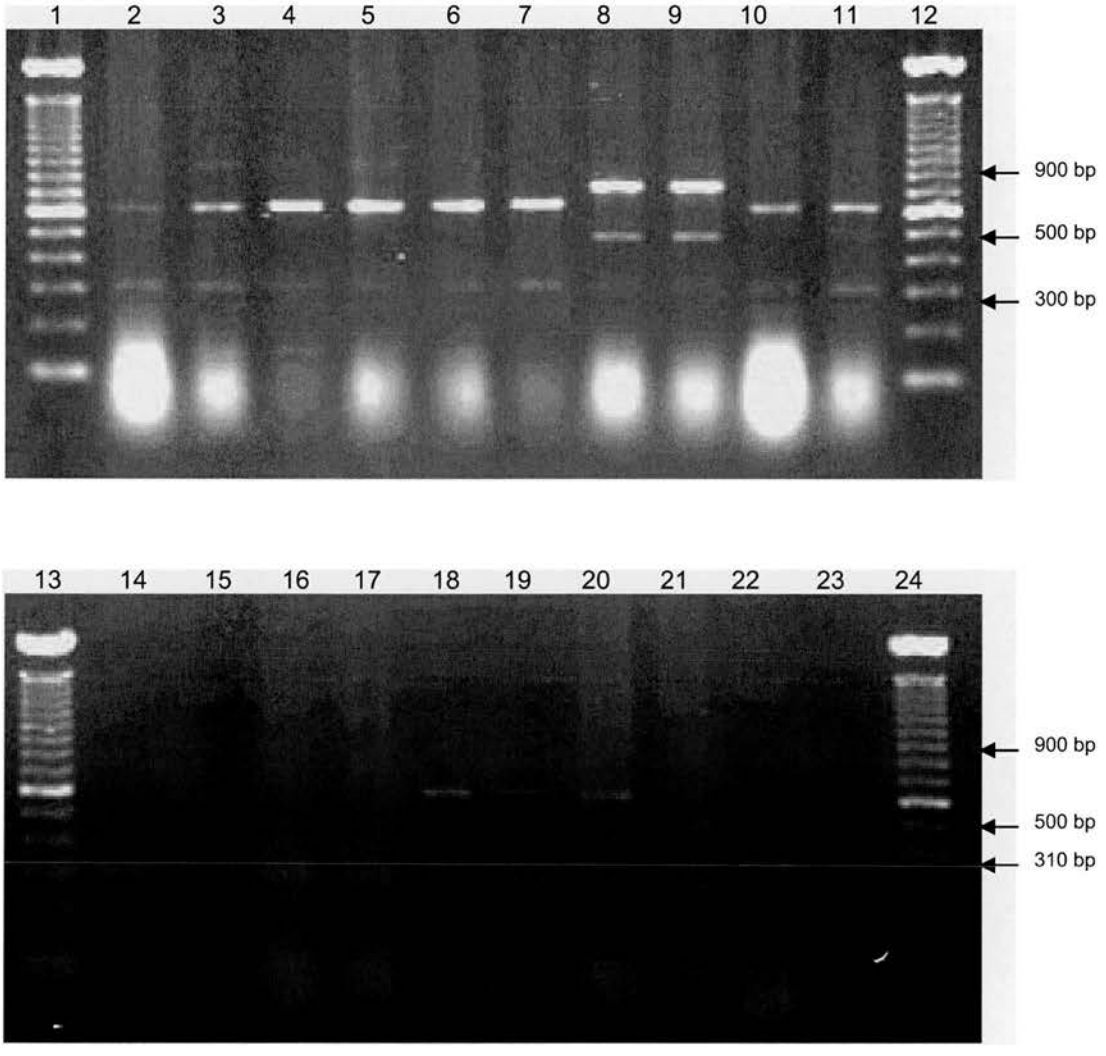

Figure 3.3. Detection of the C2CI gene by PCR in DNA from enrichment cultures from horse EBV 03/409/ Lanes 1, 12, 13 and 24, 100bp DNA ladder; lane 2, ileum day 1 enrichment; lane 3, ileum day 5 enrichment; lane 4, jejunum day 1 enrichment; lane 5, jejunum day 5 enrichment; lane 6, duodenum day 1 enrichment; lane 7, duodenum day 5 enrichment; lane 8, pharynx day 1 enrichment; lane 9, pharynx day 5 enrichment; lane 10, liver day 1 enrichment; lane 11, liver day 5 enrichment; lane 14, stomach day 1 enrichment; lane 15, stomach day 5 enrichment; lane 16, colon day 1 enrichment; lane 17, colon day 5 enrichment; lane 18, caecum day 1 enrichment; lane 19, caecum day 5 enrichment; lane 20, rectum day 1 enrichment; lane 21, rectum day 5 enrichment; lane 22 MPRL 4564 (positive standard); lanes 23 Negative control.

PCR detection of the BoNT/C gene in GS horses

PCR with the Tox384 and Tox850 primers produced unusual products of varying sizes with the DNA extracted from the enrichment cultures of whole gut samples.

A product of approximately 466bp was produced from the DNA of *C. botulinum* type C (MPRL 4564) demonstrating the presence of the BoNT/C gene and the correct working of the primers. The same result was seen when the PCR was repeated with the Tox1049 and Tox625 primers with a product of approximately 424bp produced from the DNA of *C. botulinum* type C (MPRL 4564) and multiple products of varying sizes produced from DNA extracted from whole gut enrichment cultures.

PCR using the Tox1049 and Tox384 primers was carried out on the following samples; duodenum (day 5) and spleen (day 1 and day 5) from EBV 02/352, duodenum (day 1 and day 5), colon (day 1 and day 5) and pharynx (day 5) from EBV 03/409 and duodenum (day 1 and day 5), liver (day 5) and rectum (day 1) from EBV 03/425. A total of 20 bands of varying sizes were extracted from agarose gel and re-amplified using a nested PCR reaction with primers Tox625 and Tox850 to check for single products. Sequence analysis of the 14 PCR products giving single bands gave 4 sequences that matched the gene sequence of BoNT/C when entered into a BLAST search. These sequences were obtained from the following samples; duodenum and rectum from EBV 03/425 and duodenum and pharynx from EBV 03/409. Sequence alignment using the multalin program gave 2 consensus sequences that were used to create DNA probes (Fig 3.4).

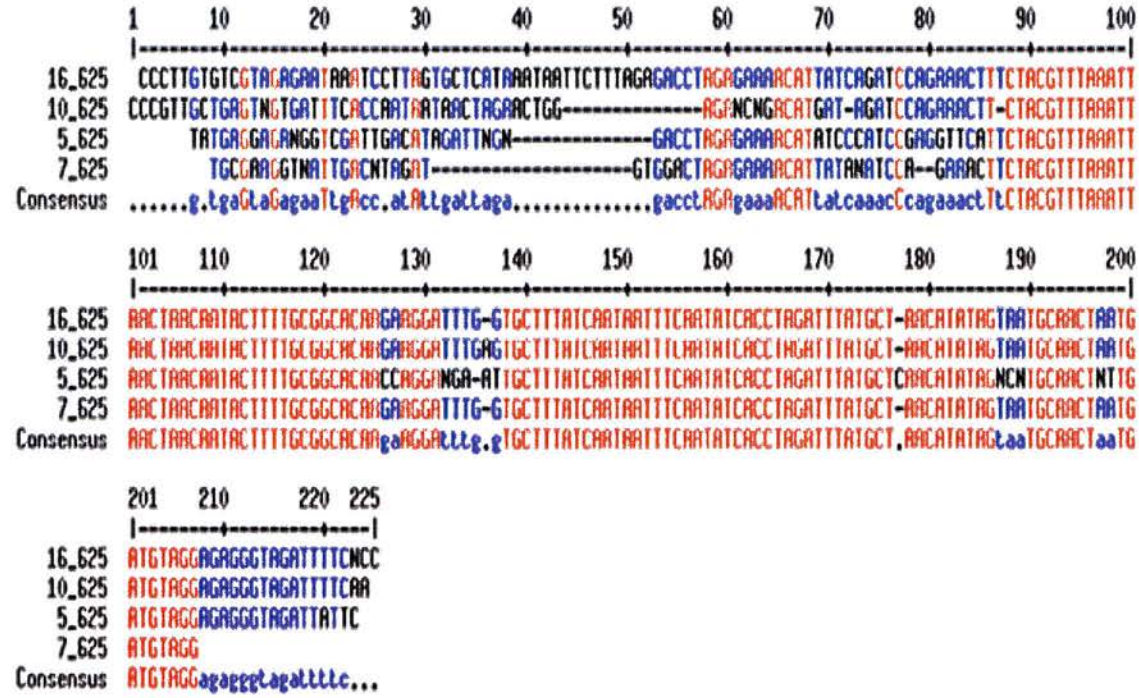

Figure 3.4. Sequence alignment of data obtained from enrichment cultures of horses with grass sickness amplified using the Tox625 primers. Sequence 16, duodenum day 1 enrichment from EBV 03/425; sequence 10, rectum day 1 enrichment from EBV 03/425; sequence 5, duodenum day 1 enrichment from EBV 03/409; sequence 7, pharynx day 5 enrichment from EBV 03/409.

PCR and Southern blot analysis of BoNT/C gene

Primary and nested PCR reactions using the Tox1049/Tox384 and Tox850/Tox625 primers respectively were run on all DNA extracted from gut enrichment cultures and the products visualised on agarose gel electrophoresis (Fig. 3.5). Of the 456 PCR products screened by Southern blotting, 13 tested positive for the presence of the BoNT/C gene (Table 3.5). This was repeated and Southern blot analysis of products separated on agarose gels was performed to confirm the positive result and to check the sizes of the products produced. Of these, 8 of the samples gave bands of approximately 450bp and 200bp as seen with the positive control and 2 produced bands of approximately 900bp.

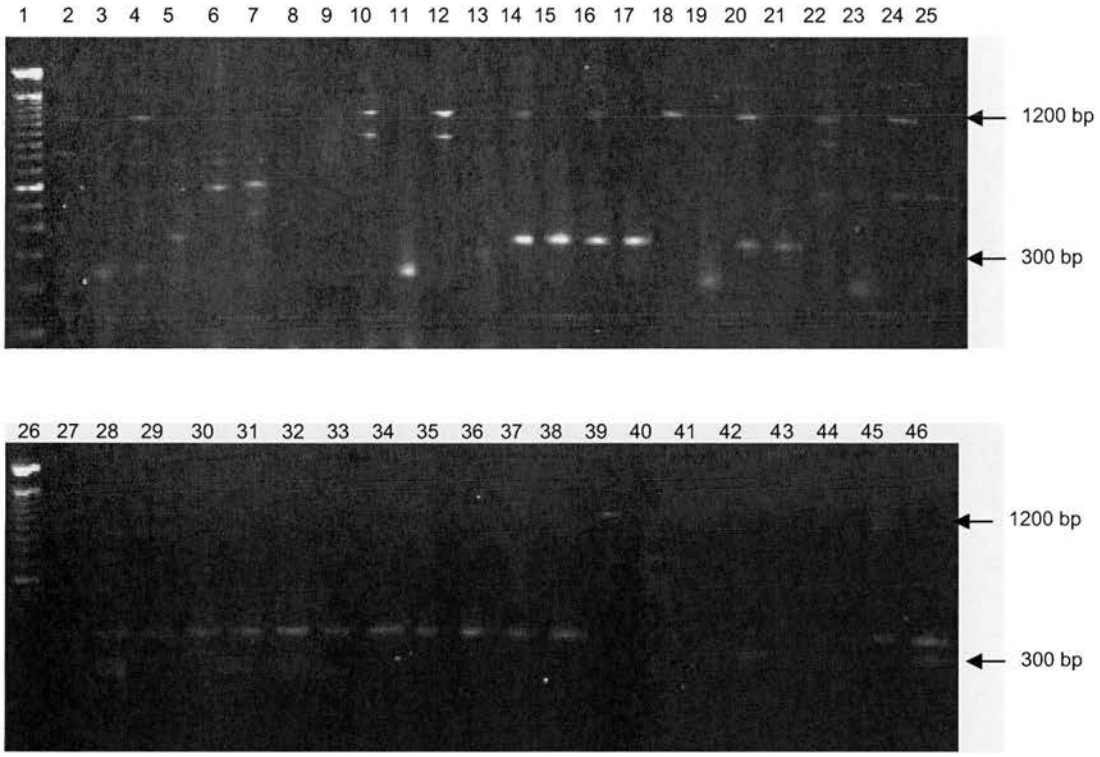

Figure 3.5a: Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 02/352. Lanes 1 and 26, 100bp DNA ladder; lane 2, ileum day 1 enrichment; lane 3, ileum day 1 enrichment nested PCR; lane 4, ileum day 5 enrichment; lane 5, ileum day 5 enrichment nested PCR; lane 6, duodenum day 1 enrichment; lane 7, duodenum day 1 enrichment nested PCR; lane 8, duodenum day 5 enrichment; lane 9, duodenum day 5 enrichment nested PCR; lane 10, spleen day 1 enrichment; lane 11, spleen day 1 enrichment nested PCR; lane 12, spleen day 5 enrichment; lane 13, spleen day 5 enrichment nested PCR; lane 14, liver day 1 enrichment; lane 15, liver day 1 enrichment nested PCR; lane 16, liver day 1 enrichment; lane 17, liver day 5 enrichment nested PCR; lane 18, pharynx day 1 enrichment; lane 19, pharynx day enrichment nested PCR; lane 20, pharynx day 5 enrichment; lane 21, pharynx day 5 enrichment nested PCR; lane 22, jejunum day 1 enrichment; lane 23, jejunum day 1 enrichment nested PCR; lane 24, jejunum day 5 enrichment; lane 25, jejunum day 5 enrichment nested PCR; lane 27, colon day 1 enrichment; lane 28, colon day 1 enrichment nested PCR; lane 29, colon day 5 enrichment; lane 30, colon day 5 enrichment nested PCR; lane 31, stomach day 1 enrichment; lane 32, stomach day 1 enrichment nested PCR; lane 33, stomach day 5 enrichment; lane 34, stomach day 5 enrichment nested PCR; lane 35, rectum day 1 enrichment; lane 36, rectum day 1 enrichment nested PCR; lane 37, rectum day 5 enrichment; lane 38, rectum day 5 enrichment nested PCR; lane 39, caecum day 1 enrichment; lane 40, caecum day 1 enrichment nested PCR; lane 41, caecum day 5 enrichment; lane 42, caecum day 5 enrichment nested PCR; lane 43, faeces day 1 enrichment; lane 44, faeces day 1 enrichment nested PCR; lane 45, faeces day 5 enrichment; lane 46, faeces day 5 enrichment nested PCR.

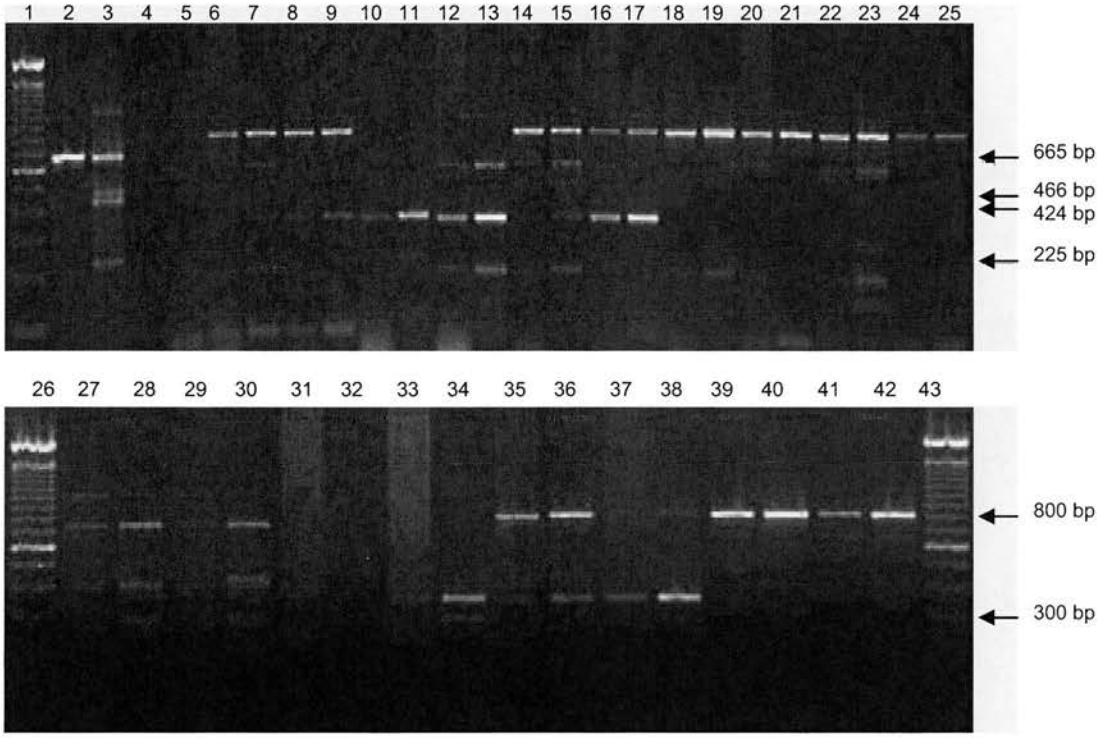

Figure 3.5b Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 03/342. Lanes 1, 26 and 43, 100bp DNA ladder; lane 2, MPRL 4564 (positive standard); lane 3, MPRL 4564 nested PCR, lanes 4-5 negative controls; lane 6, pharynx day 1 enrichment; lane 7, pharynx day 1 enrichment nested PCR; lane 8, pharynx day 5 enrichment; lane 9, pharynx day 5 enrichment nested PCR; lane 10, liver day 1 enrichment; lane 11, liver day 1 enrichment nested PCR; lane 12, liver day 5 enrichment; lane 13, liver day 5 enrichment nested PCR; lane 14, duodenum day 1 enrichment; lane 15, duodenum day 1 enrichment nested PCR; lane 16, duodenum day 5 enrichment; lane 17, duodenum day 5 enrichment nested PCR; lane 18, ileum day 1 enrichment; lane 19, ileum day 1 enrichment nested PCR; lane 20, ileum day 5 enrichment; lane 21, ileum day 5 enrichment nested PCR; lane 22, jejunum day 1 enrichment; lane 23, jejunum day 1 enrichment nested PCR; lane 24, jejunum day 5 enrichment; lane 25, jejunum day 5 enrichment nested PCR; lane 27, spleen day 1 enrichment; lane 28, spleen day 1 enrichment nested PCR; lane 29, spleen day 5 enrichment; lane 30, spleen day 5 enrichment nested PCR; lane 31, rectum day 1 enrichment; lane 32, rectum day 1 enrichment nested PCR; lane 33, rectum day 5 enrichment; lane 34, rectum day 5 enrichment nested PCR; lane 35, caecum day 1 enrichment; lane 36, caecum day 1 enrichment nested PCR; lane 37, caecum day 5 enrichment; lane 38, caecum day 5 enrichment nested PCR; lane 39, stomach day 1 enrichment; lane 40, stomach day 1 enrichment nested PCR; lane 41, stomach day 5 enrichment; lane 42, stomach day 5 enrichment nested PCR.

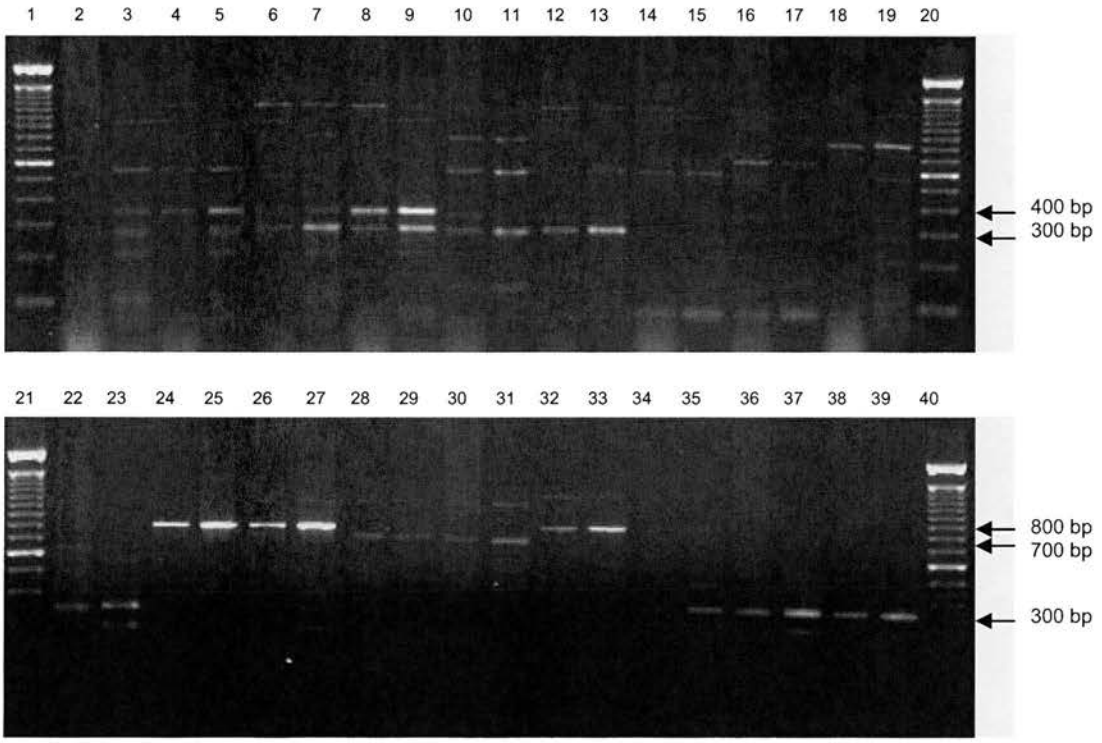

Figure 3.5c. Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 03/409. Lanes 1, 20, 21 and 40, 100bp DNA ladder; lane 2, ileum day 1 enrichment; lane 3, ileum day 1 enrichment nested PCR; lane 4, ileum day 5 enrichment; lane 5, ileum day 5 enrichment nested PCR; lane 6, jejunum day 1 enrichment; lane 7, jejunum day 1 enrichment nested PCR; lane 8, jejunum day 5 enrichment; lane 9, jejunum day 5 enrichment nested PCR; lane 10, duodenum day 1 enrichment; lane 11, duodenum day 1 enrichment nested PCR; lane 12, duodenum day 5 enrichment; lane 13, duodenum day 5 enrichment nested PCR; lane 14, pharynx day 1 enrichment; lane 15, pharynx day 1 enrichment nested PCR; lane 16, pharynx day 5 enrichment; lane 17, pharynx day 5 enrichment nested PCR; lane 18, liver day 1 enrichment; lane 19, liver day 1 enrichment nested PCR; lane22, liver day 5 enrichment; lane 23, liver day 5 enrichment nested PCR; lane 24, stomach day 1 enrichment; lane 25, stomach day 1 enrichment nested PCR; lane 26, stomach day 5 enrichment; lane 27, stomach day 5 enrichment nested PCR; lane 28, colon day 1 enrichment; lane 29, colon day 1 enrichment nested PCR; lane 30, colon day 5 enrichment; lane 31, colon day 5 enrichment nested PCR; lane 32, caecum day 1 enrichment; lane 33, caecum day 1 enrichment nested PCR; lane 34, caecum day 5 enrichment; lane 35, caecum day 5 enrichment nested PCR; lane 36, rectum day 1 enrichment; lane 37, rectum day 1 enrichment nested PCR; lane 38, rectum day 5 enrichment; lane 39, rectum day 5 enrichment nested PCR.

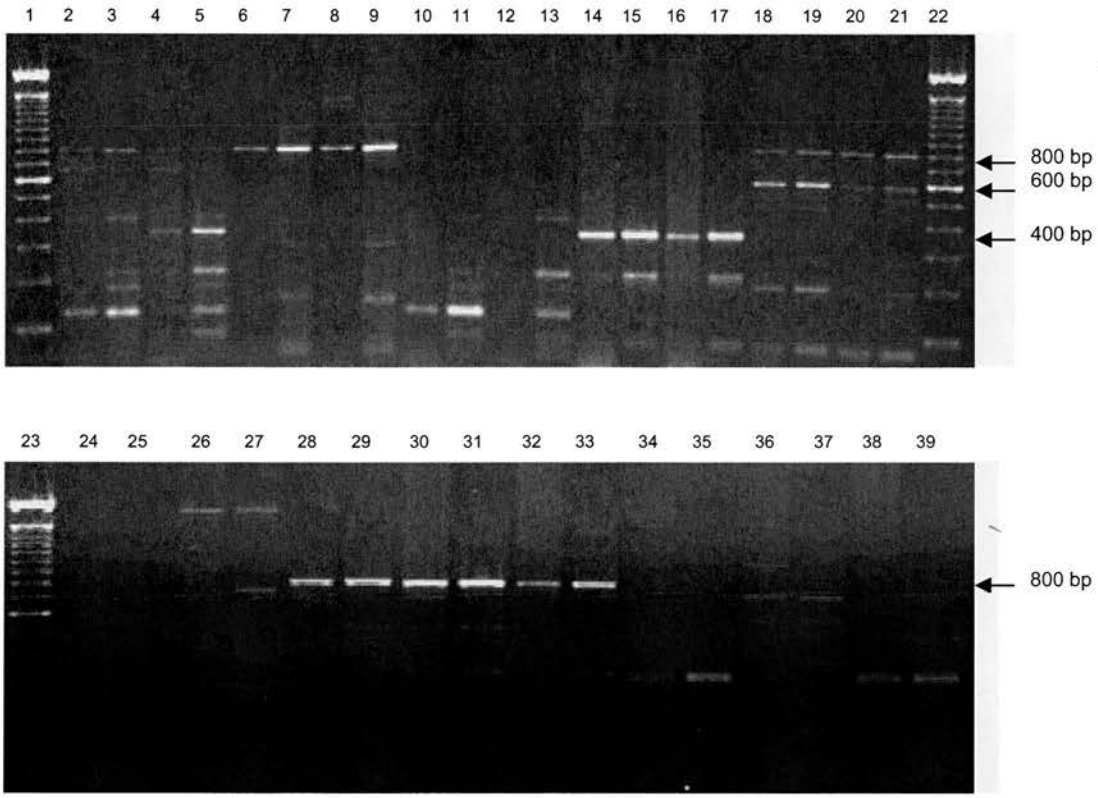

Figure 3.5d. Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 03/425. Lanes 1, 22 and 23, 100bpDNA ladder; lane 2, colon day 1 enrichment; lane 3, colon day 1 enrichment nested PCR; lane 4, colon day 5 enrichment; lane 5, colon day 5 enrichment nested PCR; lane 6, stomach day 1 enrichment; lane 7, stomach day 1 enrichment nested PCR; lane 8, stomach day 5 enrichment; lane 9, stomach day 5 enrichment nested PCR; lane 10, caecum day 1 enrichment; lane 11, caecum day 1 enrichment nested PCR; lane 12, caecum day 5 enrichment; lane 13, caecum day 5 enrichment nested PCR; lane 14, rectum day 1 enrichment; lane 15, rectum day 1 enrichment nested PCR; lane 16, rectum day 5 enrichment; lane 17, rectum day 5 enrichment nested PCR; lane 18, pharynx day 1 enrichment; lane 19, pharynx day 1 enrichment nested PCR; lane 20, pharynx day 5 enrichment; lane 21, pharynx day 5 enrichment nested PCR; lane 24, spleen day 1 enrichment; lane 25, spleen day 1 enrichment nested PCR; lane 26, spleen day 5 enrichment; lane 27, spleen day 5 enrichment nested PCR; lane 28, liver day 1 enrichment; lane 29, liver day 1 enrichment nested PCR; lane 30, liver day 5 enrichment; lane 31, liver day 5 enrichment nested PCR; lane 32, jejunum day 1 enrichment; lane 33, jejunum day 1 enrichment nested PCR; lane 34, jejunum day 5 enrichment; lane 35, jejunum day 5 enrichment nested PCR; lane 36, duodenum day 1 enrichment; lane 37, duodenum day 1 enrichment nested PCR; lane 38, duodenum day 5 enrichment; lane 39, duodenum day 5 enrichment nested PCR.

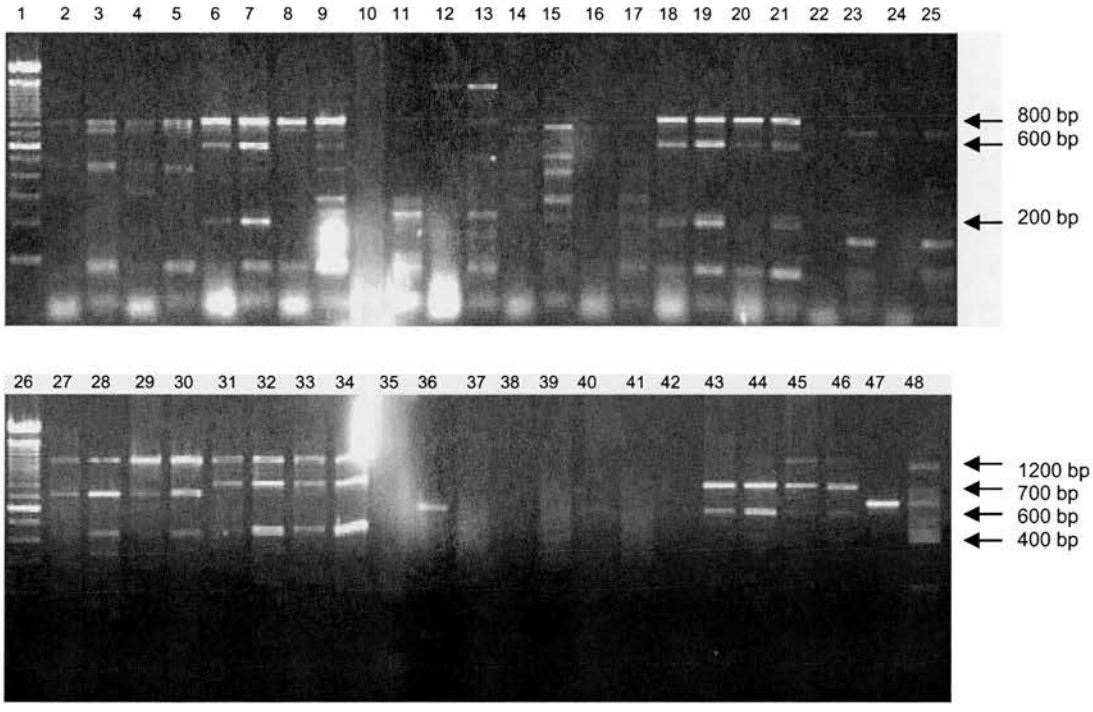

Figure 3.5e. Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 02/351. Lanes 1 and 26, 100bp DNA ladder; lane 2, ileum day 1 enrichment; lane 3, ileum day 1 enrichment nested PCR; lane 4, ileum day 5 enrichment; lane 5, ileum day 5 enrichment nested PCR; lane 6, duodenum day 1 enrichment; lane 7, duodenum day 1 enrichment nested PCR; lane 8, duodenum day 5 enrichment; lane 9, duodenum day 5 enrichment nested PCR; lane 10, liver day 1 enrichment; lane 11, liver day 1 enrichment nested PCR; lane 12, liver day 5 enrichment; lane 13, liver day 5 enrichment nested PCR; lane 14, spleen day 1 enrichment; lane 15, spleen day 1 enrichment nested PCR; lane 16, spleen day 5 enrichment; lane 17, spleen day 5 enrichment nested PCR; lane 18. pharynx day 1 enrichment; lane 19, pharynx day 1 enrichment nested PCR; lane 20, pharynx day 5 enrichment; lane 21, pharynx day 5 enrichment nested PCR; lane 22, stomach day 1 enrichment; lane 23, stomach day 1 enrichment nested PCR; lane 24, stomach day 5 enrichment; lane 25, stomach day 5 enrichment nested PCR; lane 27, colon day 1 enrichment; lane 28, colon day 1 enrichment nested PCR; lane 29, colon day 5 enrichment; lane 30, colon day 5 enrichment nested PCR; lane 31, caecum day 1 enrichment; lane 32, caecum day 1 enrichment nested PCR; lane 33, caecum day 5 enrichment; lane 34,caecum day 5 enrichment nested PCR; lane 35, faeces day 1 enrichment; lane 36, faeces day 1 enrichment nested PCR; lane 37, faeces day 5 enrichment; lane 38, faeces day 5 enrichment nested PCR; lane 39, faeces day 1 enrichment; lane 40, faeces day 1 enrichment nested PCR; lane 41, faeces day 5 enrichment; lane 42, faeces day 5 enrichment nested PCR; lane 43, jejunum day 1 enrichment; lane 44, jejunum day 1 enrichment nested PCR; lane 45, jejunum day 5 enrichment; lane 46, jejunum day 5 enrichment nested PCR; lane 47, MPRL 4564; lane 48, MPRL 4564 nested PCR.

EBV #	MPRL#	Source	PCR BoNT/C	Southern Blot BoNT/C
O3/352	3	Duodenum (day 1)	+	+
		Duodenum (day 5)	+	-
	4	Spleen (day 1)	+	+
		Spleen (day 5)	+	-
	10	Rectum (day 1)	+	+
		Rectum (day 5)	+	-
02/351	25	Ileum (day 1)	+	+
		Ileum (day 5)	+	-
	26	Duodenum (day 1)	+	+
		Duodenum (day 5)	+	+
03/409	205	Duodenum (day 1)	+	+
		Duodenum (day 5)	+	-
	206	Pharynx (day 1)	+	-
		Pharynx (day 5)	+	+
	209	Colon (day 1)	+	+
		Colon (day 5)	+	+
03/425	215	Rectum (day 1)	+	+
		Rectum (day 5)	+	-
	221	Duodenum (day 1)	+	+
		Duodenum (day 5)	+	+

Table 3.5. Detection of BoNT/C by PCR and Southern blotting in enrichment cultures. A positive PCR result denotes bands of any size detected by agarose gel electrophoresis. A positive Southern blot result denotes any sample giving a positive result for BoNT/C by dot blotting. All positive results were confirmed by repeat PCR and Southern blotting of PCR products separated by agarose gel electrophoresis.

3.1.4. Investigation of Group III clostridial genes in enrichment cultures from control horses by PCR

PCR detection of the BoNT/C gene

Primary and nested PCR reactions using the Tox1049/Tox384 and Tox850/Tox625 primers respectively gave similar banding patterns when separated by agarose gel electrophoresis as seen with the Grass Sickness horses (Fig 3.6). Southern blot analysis of the PCR products did not give any positive results indicating that the BoNT/C toxin gene was not present in any of the samples taken from control horses.

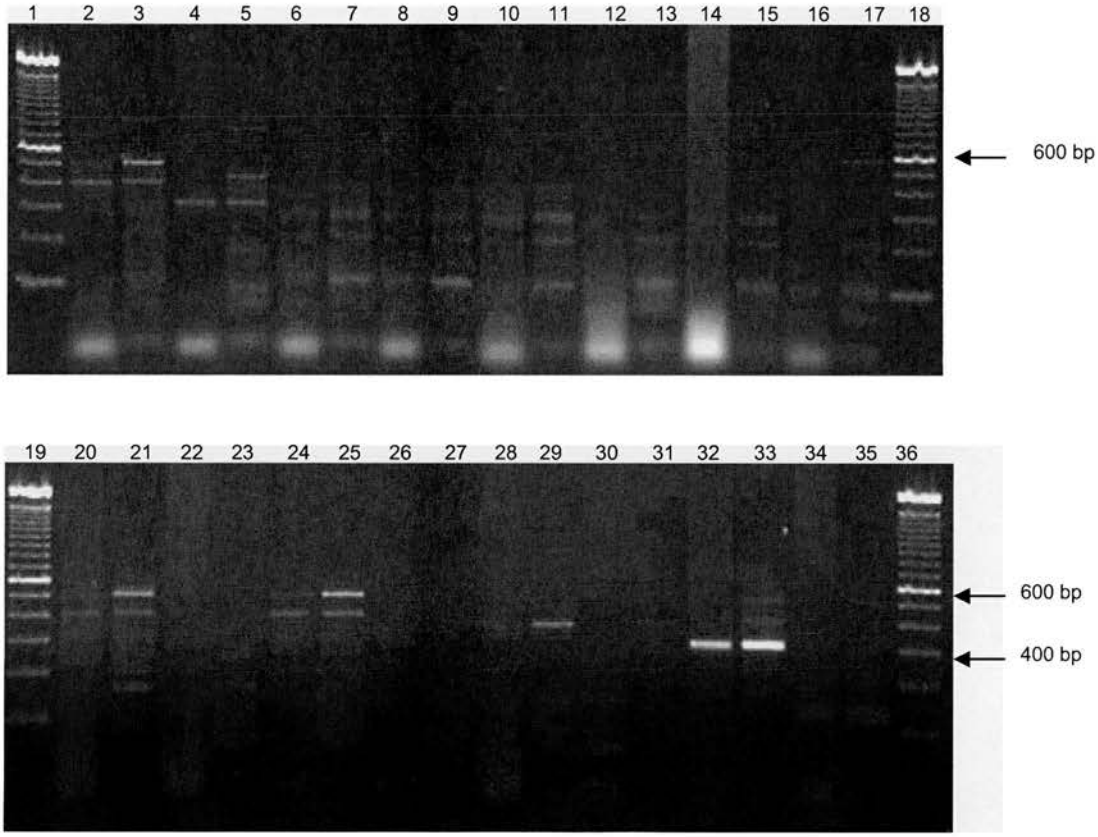

Figure 3.6a. Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 02/182. Lanes1, 18, 19 and 36, 100bp DNA ladder; lane 2, jejunum day 1 enrichment; lane 3, jejunum day 1 enrichment nested PCR; lane 4, jejunum day 5 enrichment; lane 5, jejunum day 5 enrichment nested PCR; lane 6, spleen day 1 enrichment; lane 7, spleen day 1 enrichment nested PCR; lane 8, spleen day 5 enrichment; lane 9, spleen day 5 enrichment nested PCR; lane 10, liver day 1 enrichment; lane 11, liver day 1 enrichment nested PCR; lane 12, liver day 5 enrichment; lane 13, liver day 5 enrichment nested PCR; lane 14, ileum day 1 enrichment; lane 15, ileum day 1 enrichment nested PCR; lane 16, ileum day 5 enrichment; lane 17, ileum day 5 enrichment nested PCR; lane 20, rectum day 1 enrichment; lane 21, rectum day 1 enrichment nested PCR; lane 22, rectum day 5 enrichment; lane 23, rectum day 5 enrichment nested PCR; lane 24, caecum day 1 enrichment; lane 25, caecum day 1 enrichment nested PCR; lane 26, caecum day 5 enrichment; lane 27, caecum day 5 enrichment nested PCR; lane 28, stomach day 1 enrichment; lane 29, stomach day 1 enrichment nested PCR; lane 30, stomach day 5 enrichment; lane 31, stomach day 5 enrichment nested PCR; lane 32, colon day 1 enrichment; lane 33, colon day 1 enrichment nested PCR; lane 34, colon day 5 enrichment; lane 35, colon day 5 enrichment nested PCR.

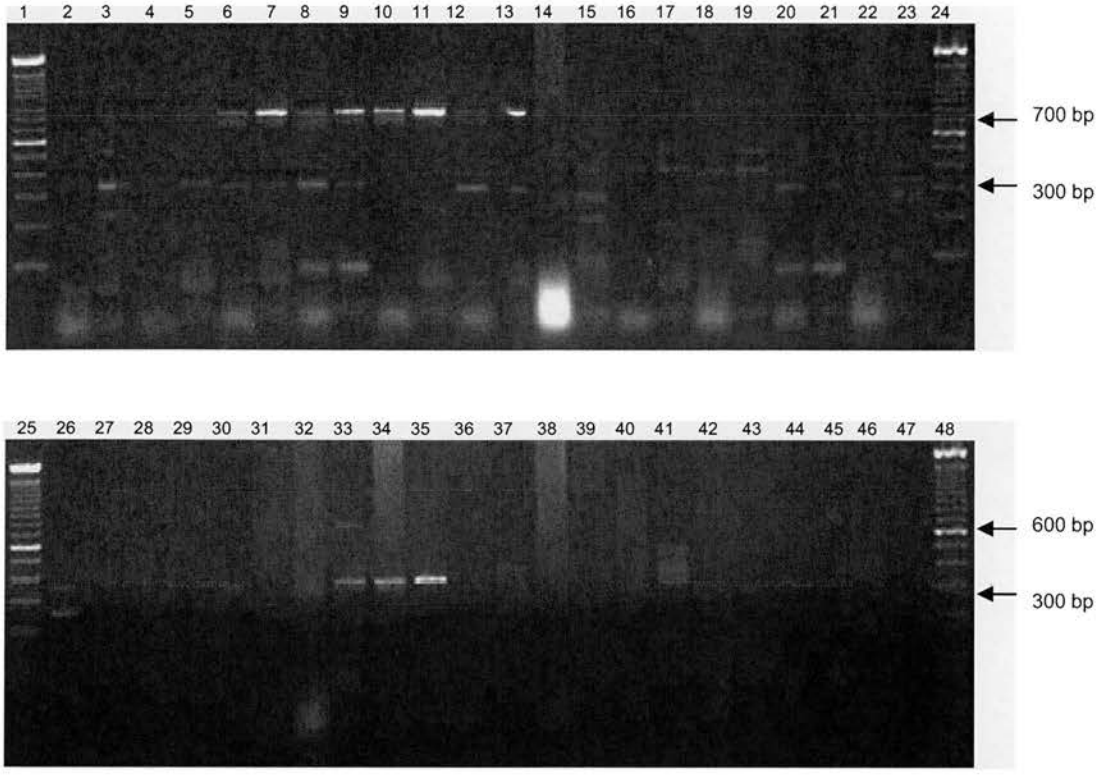

Figure 3.6b. Detection of the BoNT/C toxin gene by PCR in DNA from enrichment cultures from horse EBV 02/431. Lanes 1, 24, 25 and 48, 100bp DNA ladder; lane 2, pharynx day 1 enrichment; lane 3, pharynx day 1 enrichment nested PCR; lane 4, pharynx day 5 enrichment; lane 5, pharynx day 5 enrichment nested PCR; lane 6, ileum day 1 enrichment; lane 7, ileum day 1 enrichment nested PCR; lane 8, ileum day 5 enrichment; lane 9, ileum day 5 enrichment nested PCR; lane 10, duodenum day 1 enrichment; lane 11, duodenum day 1 enrichment nested PCR; lane 12, duodenum day 5 enrichment; lane 13, duodenum day 5 enrichment nested PCR; lane 14, liver day 1 enrichment; lane 15, liver day 1 enrichment nested PCR; lane 16, liver day 5 enrichment; lane 17, liver day 5 enrichment nested PCR; lane 18, jejunum day 1 enrichment; lane 19, jejunum day 1 enrichment nested PCR; lane 20, jejunum day 5 enrichment; lane 21, jejunum day 5 enrichment nested PCR; lane 22, spleen day 1 enrichment; lane 23, spleen day 1 enrichment nested PCR; lane 26, spleen day 5 enrichment; lane 27, spleen day 5 enrichment nested PCR; lane 28, ileum day 1 enrichment; lane 29, ileum day 1 enrichment nested PCR; lane 30, ileum day 5 enrichment; lane 31, ileum day 5 enrichment nested PCR; lane 32, stomach day 1 enrichment; lane 33, stomach day 1 enrichment nested PCR; lane 34, stomach day 5 enrichment; lane 35, stomach day 5 enrichment nested PCR; lane 36, caecum day 1 enrichment; lane 37, caecum day 1 enrichment nested PCR; lane 38, caecum day 5 enrichment; lane 39, caecum day 5 enrichment nested PCR; lane 40, colon day 1 enrichment; lane 41, colon day 1 enrichment nested PCR; lane 42, colon day 5 enrichment; lane 43, colon day 5 enrichment nested PCR; lane 44, faeces day 1 enrichment; lane 45, faeces day 1 enrichment nested PCR; lane 46, faeces day 5 enrichment; lane 47, faeces day 5 enrichment nested PCR.

PCR detection of BoNT/A, BoNT/B and BoNT/E genes

Multiplex PCR produced a 782bp product from DNA of *C. botulinum* type A strain (MPRL 2509); a 205bp product from DNA of *C. botulinum* type B strain (MPRL 2511) and a 389bp product from DNA of *C. botulinum* type E strain (MPRL 2512). PCR with the A/B/E primers did not produce a product with any of the DNA extracts from enrichment cultures indicating that none of the samples contained the BoNT/A, BoNT/B or BoNT/E genes.

3.2 Discussion

A large number of isolates were obtained from the anaerobic culture of tissue and gastrointestinal contents of horses with grass sickness and horse euthanased for non-GI related causes used as control animals. The isolates picked for purification and further study were those that resembled clostridia by colony morphology and do not represent the total microbiota of the equine gastrointestinal tract. The higher proportion of isolates obtained from horses with grass sickness has been observed previously in the faeces of horses with grass sickness (Garrett et al. 2002) though it is thought that this may reflect the intestinal stasis seen in the disease.

Seven isolates obtained from equine gastrointestinal samples of horses with grass sickness after enrichment had volatile fatty acid profiles characteristic of the Group III clostridia. A further 4 isolates resemble *C. novyi* on the basis of phenotypic characteristics. Identification of the Group III clostridia to species level requires the detection of the type-specific toxin: BoNT/C for *C. botulinum* type C, BoNT/D for

C. botulinum type D and the *C. novyi* alpha toxin for *C. novyi* type A. Each toxin is encoded by a separate bacteriophage. None of the isolates were found to contain the genes for any of the major toxins. Four isolates were found to contain the gene for one or more component of the C2 gene thought these did not resemble typical Group III organisms by phenotypic characteristics. Seven of the isolates obtained from enrichment culture of faeces from co-grazers resembled *C. novyi* by phenotype and 2 of these were found to contain genes for one or more component of the C2 toxin. None of these isolates contained the genes for any of the major toxins. Of the 25 isolates studied from control horses 12 were phenotypically characteristic of the Group III clostridia, 2 of which contained the gene for component I of the C2 toxin. A further 2 isolates contained genes for component II of the C2 toxin.

The bacteriophages encoding the toxin genes are pseudolysogenic and consequently unstable and easily lost from the host cell. Sporulation, subculturing in the laboratory and growth at 37°C can all predispose the bacterium to loss of the phage (Eklund et al, 1987). The isolates studied required repeated subculturing to obtain pure cultures. There are numerous problems involved in the culture of these organisms due to the lack of a selective medium for Group III clostridia and their fastidious nature. They are also frequently overgrown by other bacteria present in the gut. It is possible that some of the isolates obtained were producing BoNT/C or contained the genes to enable them to do so in vivo but had lost the bacteriophage carrying the BoNT/C gene by the time they were isolated in pure culture.

Identification of the organisms produced is further complicated by the large numbers of atypical organisms studied particularly in terms of the gene profiles. The reasons for this are not known though horizontal transfer of genes between clostridia has been documented. Notably strains of *C. butyricum* producing the BoNT/E toxin have been implicated in food-borne botulism (Zhou et al. 1993). Within the Group III clostridia strains of *C. botulinum* C and D have been shown to produce mosaic toxins whose antigenicity is not consistent with either BoNT/C or BoNT/D (Moriishi et al. 1996).

The C2 toxin is produced by the majority of *C. botulinum* type C strains and some *C. botulinum* type D strains. It is not known if the toxin is produced by *C. novyi* type A, but the genes have been detected in a number of *C. novyi* type A strains. The C2 toxin gene is therefore used as a marker for Group III clostridia. The genes for both components of this binary toxin are located on the bacterial chromosome and are therefore more stable then the phage-encoded toxin genes. The component I is associated with enzymatic activity and the component II with binding to target cell receptors. Many of the isolates studied were found to contain the genes for only one of the components. It is not known if this is due to the loss of one of the genes or from horizontal transfer of a single gene between organisms. It is also not known if the strains in which only the component I gene is present are capable of producing an active C2 toxin.

In all of the positive PCR results for the component I gene an unexpected number of products and bands of atypical sizes were seen. Heterogeneity in the component II

gene has been identified (Kimura et al. 1998) and strains have been divided into three groups based on differences in the molecular weight of the C2 toxin, thought to be due to differences in the gene for the component II. The primers used to amplify the component II gene had been shown to amplify DNA from all 3 groups (Kimura et al, 1998) though this study was based on a small number of reference strains.

The use of an immunomagnetic separation technique reduced the number of bacterial colonies obtained from the culture of gastrointestinal contents but did not give pure cultures of Group III clostridia. The isolates obtained showed the same atypical toxin gene profiles for their phenotype seen with the isolates obtained by traditional culture methods. Two of the isolates that were positive for the BoNT/C gene by PCR produced bands of an unexpected size and were found to produce detectable levels of toxin by ELISA-based toxin assay. These isolates were subsequently lost in subculture.

Due to the problems associated with culture and isolation of Group III clostridia and the risk of missing atypical isolates using this technique and the lack of availability of the toxin assay to confirm the presence of BoNT/C in isolates giving atypical PCR results, it was decided to concentrate on the molecular detection of toxin genes.

PCR detection of the BoNT/C gene in enrichment cultures of tissue and gastrointestinal contents again produced products of unexpected sizes. The expected product of the primary PCR reaction was a single product of 665bp with additional products of 466bp, 424bp and 225bp arising from the nested PCR reaction. An 800bp

PCR product was detected in many of the samples. Multiple bands between 100bp and 1200bp were also detected in many samples. The reasons for this are not clear though it is possible that it is due to heterogeneity in the BoNT/C gene sequence. Cross-reactivity of the primers could explain the presence of unexpected products. While the majority of bands sent for sequencing match the BoNT/C gene sequence in BLAST, matches were also found for *C. perfringens* anaerobic sulphite reductase, B subunit gene and for *Drosophila melanogaster* genes. None of these bands were detected by Southern blotting using the probes designed from consensus sequences of the BoNT/C products from gastrointestinal samples.

The BoNT/C gene was detected in 4 of the 7 horses with histopathologically confirmed grass sickness by Southern blotting. None of the control horses were found to contain the BoNT/C gene. This may be due to the small number of genes/isolates in the GI tract and the small area sampled for culture. All of the samples that gave a positive result by Southern blotting showed a banding pattern consistent with the *C. botulinum* type C (MPRL 4564) used as a positive control and product sizes were as expected with the primers used. Southern blotting also appeared to enable the detection of bands present in the DNA extracts from enrichment cultures that were invisible under uv light on agarose gels stained with ethidium bromide.

Where the BoNT/C gene was found in equine samples, it was consistently detected in the duodenal contents. In addition, the BoNT/C gene was detected in the rectal contents of two of the animals in the study. Distribution of the toxin gene along the

GI tract in acute cases may explain the neurological degeneration seen at most sites along the gastrointestinal tract sampled in acute cases (Scholes et al. 1993; Doxey et al. 1995). The detection of the BoNT/C gene in the pharynx sample may be a result of cross-contamination during sample collection. The size of the pharynx was too small to allow the outer part of the sample to be discarded as in other samples. Of the 13 enrichment cultures that tested positive for BoNT/C by PCR and Southern blotting 9 were from overnight incubation and only 4 from 5 day incubation. This may indicate the presence of free bacteriophage in the contents of the gastrointestinal tract that is degraded during the incubation period.

The results obtained may provide further evidence of a link between *C. botulinum* type C and equine grass sickness when combined with the serological data. Significantly lower levels of IgG to both the surface antigens of Group III clostridia and to BoNT/C have been detected in horses with grass sickness compared to controls (Hunter and Poxton, 2001). It is not known if this difference is due to the *in vivo* neutralisation of the organism and toxin resulting in lower levels of antibodies available for detection, or if the horses with grass sickness would have demonstrated lower levels of IgG prior to the onset of the disease, allowing the proliferation and toxin production of *C. botulinum* type C within the gastrointestinal tract. The detection of significantly higher levels of IgA to BoNT/C within the gastrointestinal tract of horses with grass sickness compared to controls suggests recent exposure to the toxin in animals that develop the disease (Nunn et al, 2007).

The detection of C2 toxin genes in control animals as well as those with grass sickness may support the hypothesis that animals carry a Group III like organism within the gut that either carries the phage or becomes infected with the phage within the GI tract. The inability to isolate characteristic organisms and the detection of toxin genes from whole gut enrichment cultures suggests that a molecular approach is needed. The use of PCR for the detection of Group-III specific 16s rRNA or similar genes would enable the detection of carriage of these organisms in both affected and healthy horses.

Chapter Four

Molecular Characterisation and detection of toxin genes of Group III clostridia

Due to the detection of isolates of Group III clostridia containing genes for one or more component of the C2 toxin and none of the defining major toxins, the following work was carried out to investigate the toxin gene profiles of a variety of Group III clostridia from human, environmental and animal sources. Much of the material presented in this chapter has been published (See Appendix)

4.1 Results

4.1.1 Investigation of Group III toxin genes by PCR

PCR detection of the novyi alpha toxin gene

All of the strains studied were found to be phenotypically similar and consistent with the Group III clostridia producing both lecithinase and lipase when grown on egg yolk agar. All were microscopically identified as Gram positive or Gram variable rods. Spores, if present, were large and sub-terminal. Twenty two out of forty strains identified as *C. novyi* type A carried the alpha toxin gene (Table 4.1), as demonstrated by the presence of a 260bp PCR product, using the Nov primers (Fig. 4.1). None of the strains identified as *C. botulinum* type C or type D were found to carry the alpha toxin gene.

PCR detection of the BoNT/C and BoNT/D genes

PCR with the Tox-384/Tox-850 primers produced a product of 466bp in one of the strains identified as *C. botulinum* type C (NCTC 8548) demonstrating the presence of the BoNT/C gene. The lack of PCR product in the other two type strains (NCTC 3732 and NCTC 10914) suggests that the phage carrying the BoNT/C gene has been lost. These strains had previously been shown to be BoNT/C negative by use of a specific ELISA for the direct detection of the toxin. None of the organisms identified as *C. novyi* type A or *C. botulinum* type D were found to carry the BoNT/C gene by PCR.

PCR with the ToxD primers produced a 497bp product from DNA of *C. botulinum* type D strain (NCTC 8265) demonstrating the presence of the BoNT/D gene. None of the organisms identified as *C. novyi* type A or *C. botulinum* type C were found to be carrying the BoNT/D gene.

PCR detection of the C2 toxin genes

Thirty one of the strains carried the gene for the component I of the C2 toxin (Table 4.1), as demonstrated by the production of a 310bp product using the C2CI primers. The *C. botulinum* type D strain (MPRL 3923) produced a PCR product of approximately 650bp with the C2CI primers (Fig 4.2). Sequence analysis of this product showed it to match the sequence of the C2 toxin gene when entered into a BLAST search.

Eight of the strains produced a PCR product of 1049bp using the C2CII primers indicating the presence of the gene for the component II of the C2 toxin.

Only five of the strains carried genes for both the CI and CII components of the C2 toxin; the *C. botulinum* type C strain (MPRL 4564) which produces the BoNT/C and the non neurotoxin producing strain MPRL 2510; the *C. botulinum* type D strain (MPRL 3923) and two of the strains identified as *C. novyi* type A (MPRL 2307 and 2534) both of which were found to be negative for the novyi alpha toxin gene by PCR.

Lab No.	Organism	novyi α	Toxin C2CI	Profile C2CII	By PCR BoNT/C	BoNT/D
141	*C. novyi* type A-like	-	+	-	-	-
465	*C. novyi* type A	-	+	-	-	-
2306	*C. novyi* type A	-	+	-	-	-
2307	*C. novyi* type A	-	+	+	-	-
2510	*C. botulinum* type C	-	+	+	-	-
2530	*C. novyi* type A	+	+	-	-	-
2531	*C. novyi* type A	+	+	-	-	-
2533	*C. novyi* type A	-	+	-	-	-
2534	*C. novyi* type A	-	+	+	-	-
2535	*C. novyi* type A	-	+	-	-	-
2536	*C. novyi* type A	+	+	-	-	-
3209	*C. novyi* type A	+	+	-	-	-
3341	*C. novyi* type A	-	-	-	-	-
3923	*C. botulinum* type D	-	+	+	-	+
4540	*C. novyi* type A	+	+	-	-	-
4541	*C. novyi* type A	+	+	-	-	-
4545	*C. novyi* type A	+	+	-	-	-
4547	*C. novyi* type A	+	+	-	-	-
4548	*C. novyi* type A	+	+	-	-	-
4549	*C. novyi* type A	-	+	-	-	-
4550	*C. novyi* type A	-	-	-	-	-
4551	*C. novyi* type A	-	+	-	-	-
4552	*C. novyi* type A	+	-	-	-	-
4553	*C. novyi* type A	-	-	-	-	-
4554	*C. novyi* type A	-	+	-	-	-
4556	*C. novyi* type A	-	-	-	-	-
4557	*C. novyi* type A	-	-	-	-	-
4558	*C. novyi* type A	-	+	-	-	-
4559	*C. novyi* type A	-	-	-	-	-
4561	*C. novyi* type A	-	+	-	-	-
4562	*C. novyi* type A	+	+	-	-	-
4563	*C. novyi* type A	+	+	-	-	-
4564	*C. botulinum* type C	-	+	+	+	-
4565	*C. botulinum* type C	-	+	-	-	-
4566	*C. novyi* type A	+	+	-	-	-
4567	*C. novyi* type A	+	+	-	-	-
4568	*C. novyi* type A	+	+	-	-	-
4569	*C. novyi* type A	+	+	-	-	-
4570	*C. novyi* type A	+	-	-	-	-
4571	*C. novyi* type A	+	+	-	-	-
4572	*C. novyi* type A	+	-	-	-	-
4573	*C. novyi* type A	+	-	+	-	-
4574	*C. novyi* type A	+	-	+	-	-
4575	*C. novyi* type A	+	-	+	-	-

Table 4.1: Toxin gene profile of Group III clostridia from the laboratory collection.

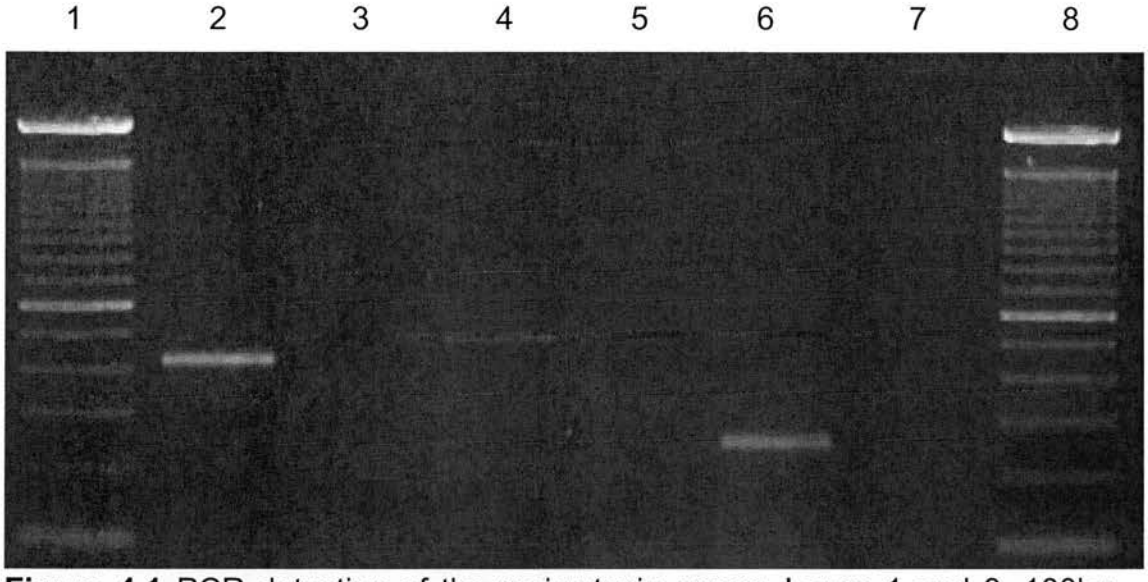

Figure 4.1 PCR detection of the major toxin genes. Lanes 1 and 8, 100bp ladder; lanes 2 and 3, BoNT/C, strain 4564 and negative control; lanes 4 and 5 BoNT/D, strain 3929 and negative control and lanes 6 and 7 *C. novyi* α toxin, strain 2307 and negative control.

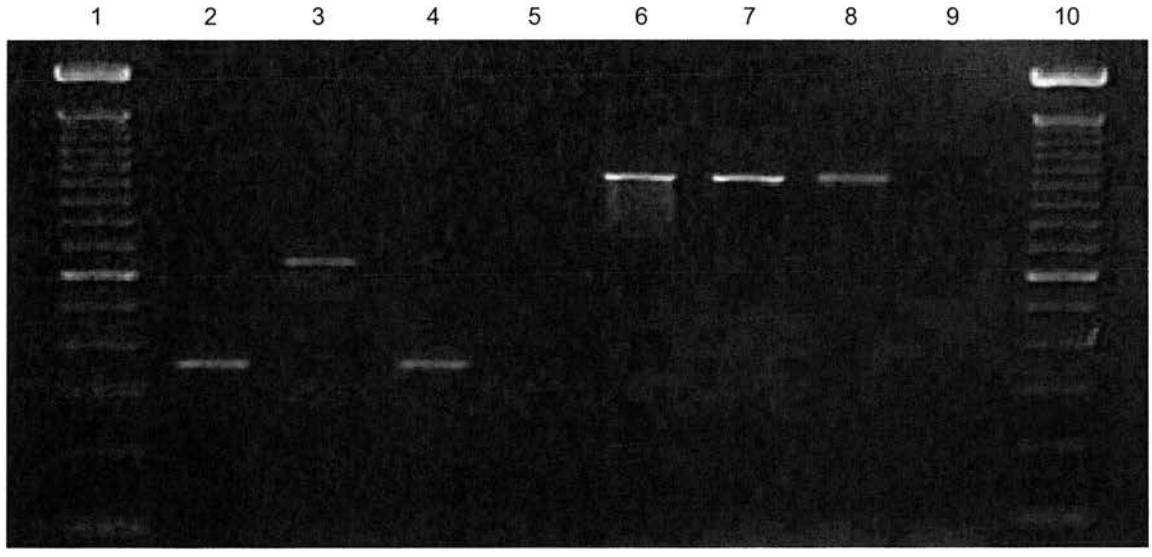

Figure 4.2 PCR detection of the C2 toxin genes. Lanes 1 and 10, 100bp ladder; lanes 2-5, C2CI strains 4564, 3923, 2307 and negative control; lanes 6-9 C2CII strains 4565, 3923, 2307 and negative control.

4.1.2 Investigation of the 16S rRNA gene in Group III clostridia

All of the strains produced a PCR product of approximately 400bp with the 16S primers (Fig 4.3) demonstrating the presence of the bacterial 16s ribosomal RNA gene and the correct working of the primers.

A heteroduplex mobility assay was devised in order to detect any difference in the sequence of the 16s rRNA gene within the Group III clostridia in an attempt to further identify or group them at a molecular level. Additional bands were seen when this was carried out with duplexes between *C. botulinum* type C (strain 4564) and a range of clostridia (Fig 4.4) indicating possible difference between the DNA sequence within a section of the gene. No additional bands were seen with duplexes between *C. botulinum* type C (Strain 4564) and the other Group III clostridia (Fig. 4.5) which suggests a greater degree of similarity in the gene sequence of the portion of the 16s rRNA gene studied.

Sequence analysis of the 16s rRNA gene in Group III clostridia

Sequence analysis of the purified PCR products confirmed that all sequences matched the sequence of the 16S rRNA gene in BLAST. Sequence alignment of the data obtained showed a high degree of homology between the sequences of the Group III clostridia tested (Fig 4.6), a lower degree of sequence homology was observed between the gene sequences obtained from the unrelated clostridia (Fig 4.7). These observations are consistent with the results seen in the heteroduplex mobility assay studies.

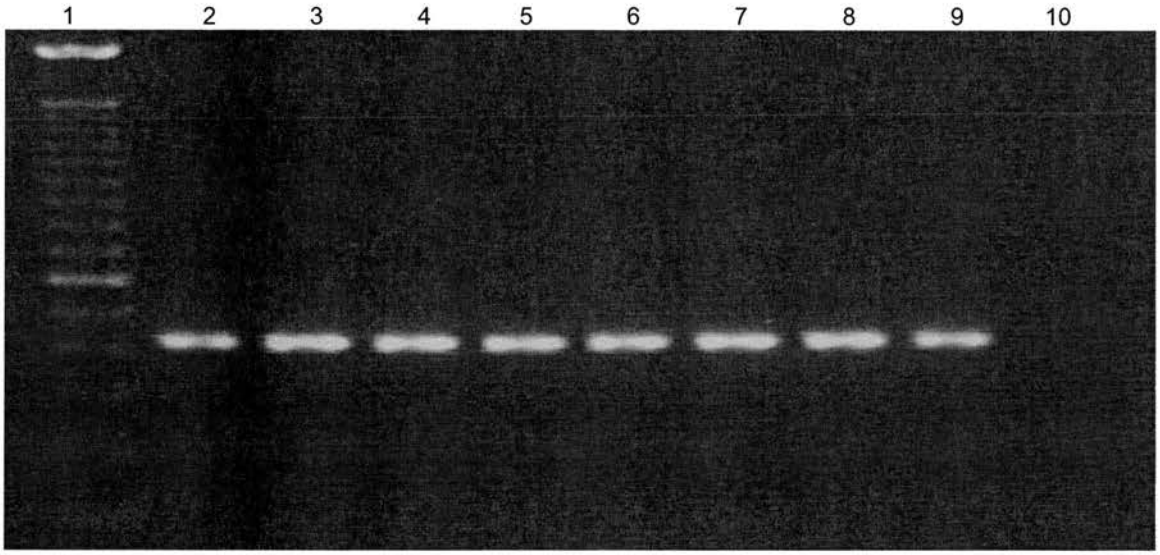

Figure 4.3 PCR detection of the 16S rRNA gene. Lane 1, 100bpDNA ladder; lane 2, MPRL 141; lane 3, MPRL 2307; lane 4, MPRL 2510; lane 5 MPRL 2530; lane 6, MPRL 3923; lane 7, MPRL 4550; lane 8, MPRL 4564; lane 9, MPRL 4575; lane 10, negative control.

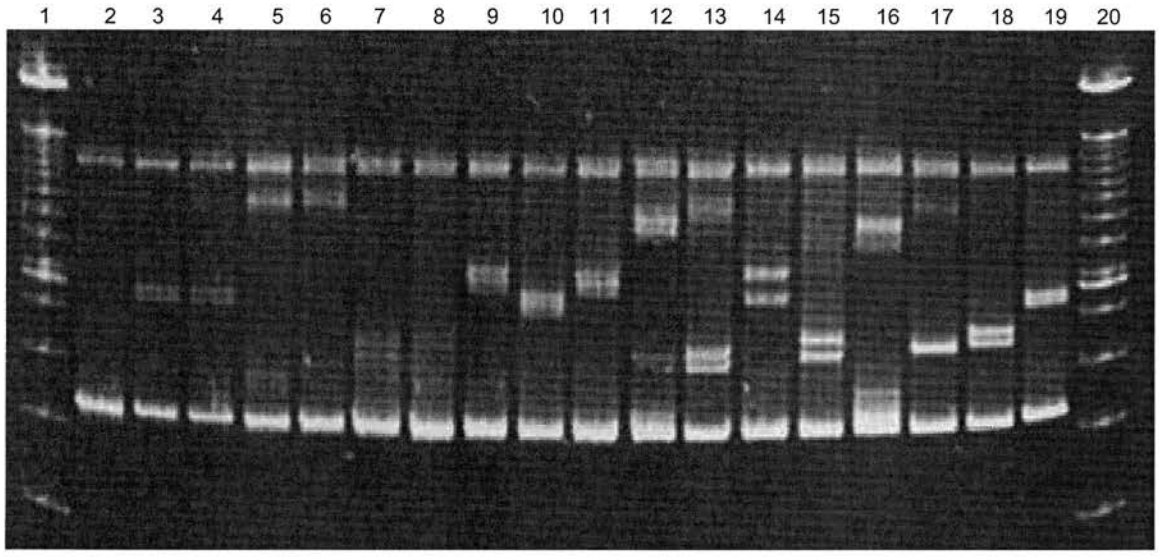

Figure 4.4 Heteroduplex mobility assay of 16s rRNA PCR products. Lanes 1 and 20, 100bp ladder; lane 2, MPRL 4564; lane 3, MPRL 4564 and MPRL 2509; lane 4, MPRL 4564 and MPRL 2511; lane 5, MPRL 4564 and MPRL 2512; lane 6, MPRL 4564 and MPRL 2661; lane 7, MPRL 4564 and MPRL 2778; lane 8, MPRL 4564 and MPRL 3923; lane 9, MPRL 4564 and MPRL2645; lane 10, MPRL 4564 and MPRL2591; lane 11, MPRL 4564 and MPRL 4354; lane 12, MPRL 4564 and MPRL 4526; lane 13, MPRL 4564 and MPRL 2618; lane 14, MPRL 4564 and MPRL 2528; lane 15, MPRL 4564 and MPRL 2656; lane 16, MPRL 4564 and MPRL 2651; lane 17, MPRL 4564 and MPRL 3271; lane 18 MPRL 4564 and MPRL 3573; lane 29, MPRL 4564 and *E. coli*.

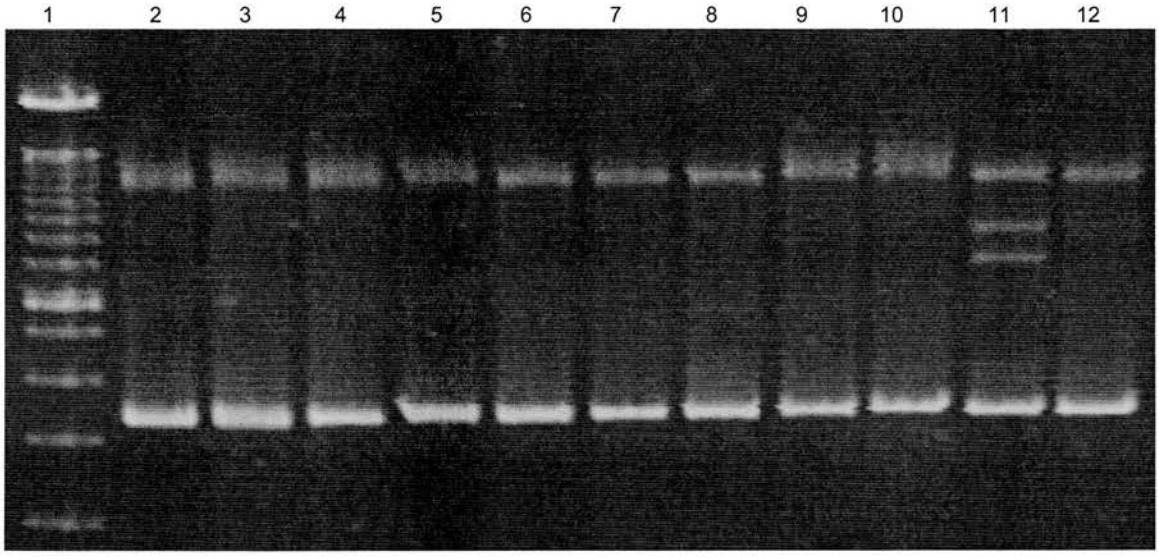

Figure 4.5 Heteroduplex mobility assay of 16S rRNA PCR products. Lane 1, 100bp ladder; lane 2 MPRL 4564 and MPRL 141; lane 3, MPRL 4564 and MPRL 2533; lane 4 MPRL 4564 and MPRL 3923; lane 5 MPRL 4564 and MPRL 3209; lane 6, MPRL 4564 and MPRL 4545; lane 7, MPRL 4564 and MPRL 4550; lane 8, MPRL 4564 and MPRL 4552; lane 9, MPRL 4564 and MPRL 4558; lane 10, MPRL 4564 and MPRL 4565; lane 11, MPRL 4564 and *Staph. aureus*; lane 12, MPRL 4564.

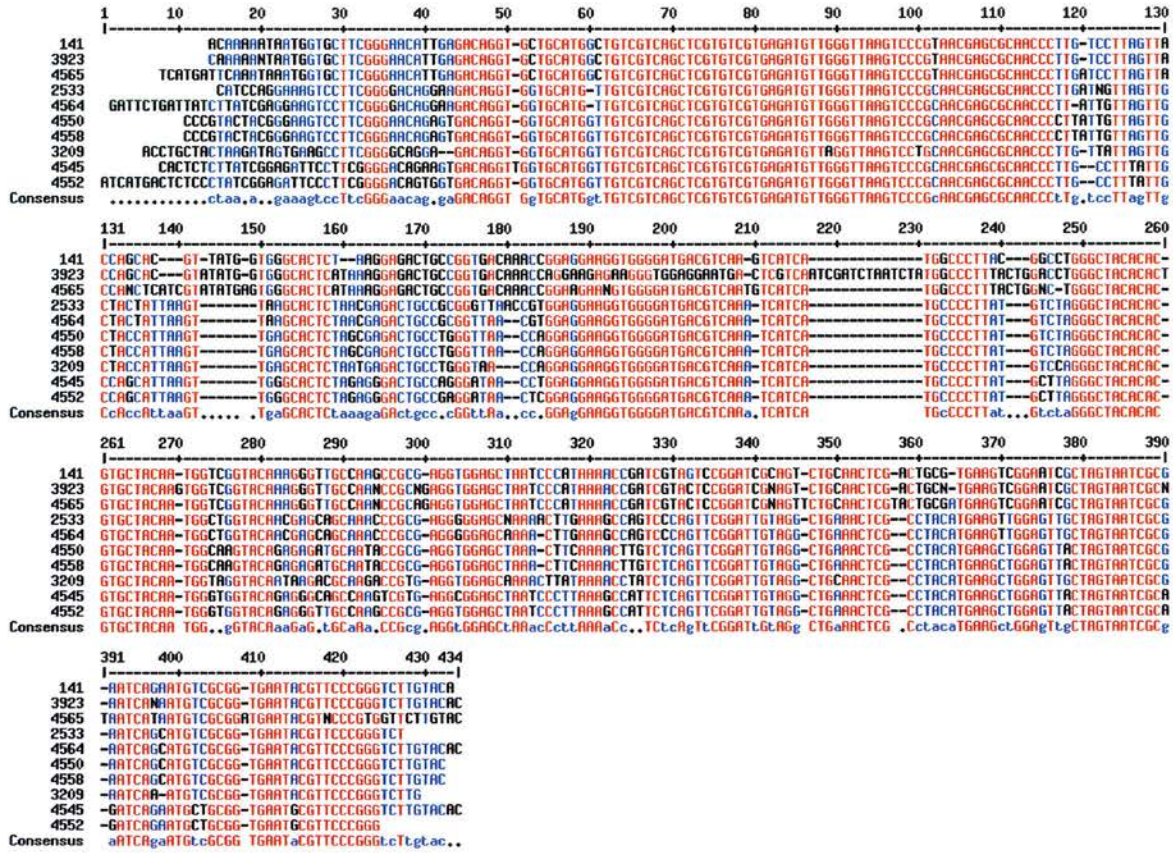

Figure 4.6: Sequence alignment data obtained from Group III clostridia using the 16S primers. Blue text denotes 50% sequence homology and red text denotes 90% sequence homology.

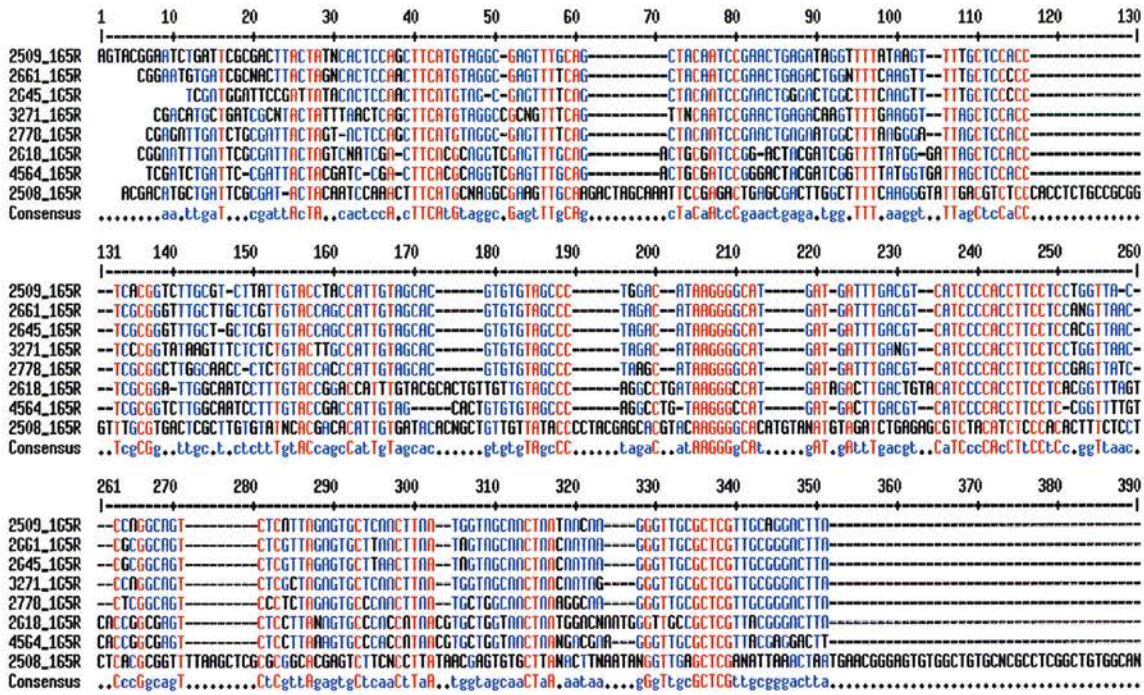

Figure 4.7: Sequence alignment data obtained from clostridia using the 16S primers. Blue text denotes 50% sequence homology and red text denotes 90% sequence homology.

4.1.3 Investigation of the rpoB gene in Group III clostridia

All of the isolates tested were found to contain the rpoB gene producing a PCR product between 300bp and 400bp demonstrating the presence of the bacterial rpoB gene and the correct working of the primers. Heteroduplex mobility assay analysis of the PCR products obtained from the Group III clostridia tested revealed no difference in the banding patterns obtained suggesting a high degree of homology in the region of the gene tested.

4.2 Discussion

Of the 40 strains previously identified as *C. novyi* type A by phenotypic methods, only 22 were found to contain the *C. novyi* α toxin gene by PCR. It is possible that this may be due to mis-match of the primers or heterogeneity in the sequence of the *C. novyi* α toxin gene. Further sequencing of several α toxin genes would be required to ensure that the primers are sensitive enough to detect the gene. The pseudolysogenic relationship between the bacterial cell and bacteriophage is inherently unstable. It is postulated that cycles of phage loss and re-infection occur in nature. It is known that sub-culturing in the laboratory pre-disposes to phage loss as does sporulation and culture of the organism at 37°C (Eklund et al, 1987). As many of the strains tested have been part of the laboratory culture collection for many years it is possible that they have lost the novyi α toxin gene at some point.

All of the strains tested that had been isolated from cases of infection in IDUs were found to carry the *C. novyi* α toxin gene. All of these cases showed characteristic pathology consistent with the action of the novyi α toxin. None of the isolates obtained from IDU's appear to contain genes for both components of the C2 toxin with the majority (64%) containing the gene for the enzymatic component (C2CI) only, 27% containing the gene for the transport component (C2CII) only and one isolate containing no C2 genes. It is not known if the genes are expressed by the bacteria, reverse-transcriptase PCR would be necessary to determine this. It is possible that the enzymatic component may play a part in the pathology and that the transport component (C2CII) may not be required for the transport of the active component into damaged cells.

The distribution of toxin genes in animal and environmental samples appears to be different with only 3 of the 14 strains tested carrying the novyi α toxin gene though all of the isolates are phenotypically similar. The distribution of the C2 toxin genes is similar in the animal and environmental isolates with 43% containing the gene for the C2CI and none containing the gene for the C2CII. Only five strains were found to contain the genes for both components of the C2 toxin, both the neurotoxin producing *C. botulinum* type strains 4565 (type C) and 3923 (type D); one of the BoNT/C-negative *C. botulinum* type C strains and two of the strains classed as *C. novyi* type A that were found to be negative for the novyi α toxin. The origin of these strains is unknown and it is possible that they are also *C. botulinum* strains that have lost the neurotoxin gene.

Of the strains in which no toxin genes were detected, 5 were isolated from animals; one from a hare with dysautonomia and four from horses, two of which had active dysautonomia at the time of sampling. The other was isolated from a soil sample.

The C2 toxin genes are carried on the bacterial chromosome and the toxin is produced by the majority of *C. botulinum* type C strains and by some *C. botulinum* type D strains (Fujii et al, 1996; Kimura et al, 1998), though it had not been reported as being produced by *C. novyi* type A. The presence of the genes for one or more component of the C2 toxin gene in isolates may provide evidence of the horizontal transfer of genetic material between Group III clostridia in vivo. This further demonstrates the close relationship between the Group III clostridia and the difficulty

in identifying organisms when the major toxin is not present. This close relationship is also seen in the high degree of homology in the 16S rRNA gene as demonstrated by sequence analysis of PCR products from the Group III clostridia investigated, compared with the lower degree of sequence homology between unrelated clostridia.

It is possible that the low level of detection of the component II gene may be due to sequence differences between the organisms. Heterogeneity in the component II gene has been identified (Kimura et al, 1998) and strains have been divided into three groups based on differences in the molecular weight of the C2 toxin, thought to be due to differences in the gene for the component II. The published primers had been shown to amplify the component II gene from all three groups but were found to produce inconsistent results and were re-designed based on a published sequence (Kimura et al, 1998). It is possible that the lack of detection is therefore due to differences in the gene structure meaning that they are not detected by the primers. In order to verify this more sequence data would be needed from several isolates.

The investigation of C2 toxin production in strains in which one or both of the C2 toxin gene components is required to establish whether these genes are no longer present or whether the PCR is not sensitive enough to detect the differences in gene sequences. This would require sequencing of several strains in order to produce primers for reverse-transcriptase PCR. Antibodies for the direct detection of the CI and CII components of the C2 toxin by dot blotting were investigated but were found to be highly cross-reactive with a number of different clostridia.

It has been hypothesised that a common bacterial strain exists in nature and that its pathogenicity is determined by the toxin it produces and therefore by the presence of the specific converting phage (Eklund et al, 1974). There is therefore a question as to whether the Group III clostridia should be classified as separate species. The only method of differentiating between the organisms relies on the production of the major toxin that is carried on a bacteriophage. However, the bacteriophages are pseudolysogenic and highly unstable. They are also potentially able to infect all three species of clostridia, effectively changing the identity of the cell they infect. It had been thought that the detection of the minor toxins such as the C2 toxin could be used to differentiate between *C. botulinum* and *C. novyi* strains, particularly those that have lost the phage encoding the major toxin. However, this study has demonstrated that the toxin gene profiles of these organisms are highly variable and further demonstrates how closely related these organisms are.

Chapter Five

Investigation into the prevalence of toxigenic clostridia in seabirds

The techniques developed for the detection of botulinum toxin genes from GI enrichment cultures were used to investigate the prevalence of botulinum neurotoxin genes in sea birds and their potential as a reservoir for infection. The association between the Fulmar and neonatal tetanus was also investigated in this chapter.

5.1 Results

5.1.1 Direct detection of BoNT/C in the gastrointestinal contents and plasma of seabirds by ELISA

Of the ten samples tested for the presence of BoNT/C by ELISA, 5 were found to contain detectable levels of the toxin (Table 5.1). Of these, two showed an increase in the level of toxin detected over the 5 day incubation period of the enrichment (B303427/1 and B303467). This indicated the presence of viable bacteria in the sample that continued to produce neurotoxin. A decrease in the amount of toxin detected over the five day culture period was seen in three of the samples (B303534/2; B303427/4 and B303515/1). This suggested that viable cells were no longer present in the sample or that any cells present were no longer producing neurotoxin.

Sample Number	Sample type	ELISA toxin (ng/ml)	
		Day 0	Day 5
B303427/1	GI contents (herring gull)	1.8	9.8
B303427/2	GI contents (herring gull)	<0.8	<0.8
B303467	GI contents (gannet)	1.4	6.0
B303467b	Plasma (gannet)	<0.8	<0.8
B303515/1	GI contents (herring gull)	4.0	1.2
B303515/2	GI contents (herring gull)	<0.8	<0.8
B303427/3	Plasma (herring gull)	<0.8	<0.8
B303427/4	Plasma (herring gull)	1.07	<0.8
B303534/2	GI contents (herring gull)	4.0	3.0
B303534/2b	Plasma (herring gull)	<0.8	<0.8

Table 5.1: Direct detection of BoNT/C by ELISA in gastrointestinal contents and plasma samples from seabirds.

5.1.2 Detection of botulinum neurotoxin types A-E genes by PCR

Detection of the BoNT/A, BoNT/B and BoNT/E genes by multiplex PCR

The multiplex PCR assay used was found to give inconsistent results when all three primer sets were used with a high incidence of false negatives in the detection of the BoNT/E gene. It was therefore decided to use the BoNT/A and BoNT/B primer sets only in the multiplex PCR reaction and perform the PCR for the detection of the BoNT/E gene separately.

PCR with the multiplex primers produced a 782bp PCR product with DNA from the *C. botulinum* type A strain (MPRL 2509) (Figure 5.1) demonstrating the presence of the BoNT/A gene. Seven of the 20 DNA samples taken from the overnight culture of the gastrointestinal contents of seabirds also produced a 782bp PCR product indicating the presence of the BoNT/A gene within the GI contents of those birds (Table 5.2).

A PCR product of 205bp was amplified from the *C. botulinum* type B strain (MPRL 2511) (Figure 5.1) demonstrating the presence of the BoNT/B gene. Eight of the 20 DNA extracts from enrichment cultures were also found to contain the BoNT/B gene (Table 5.2).

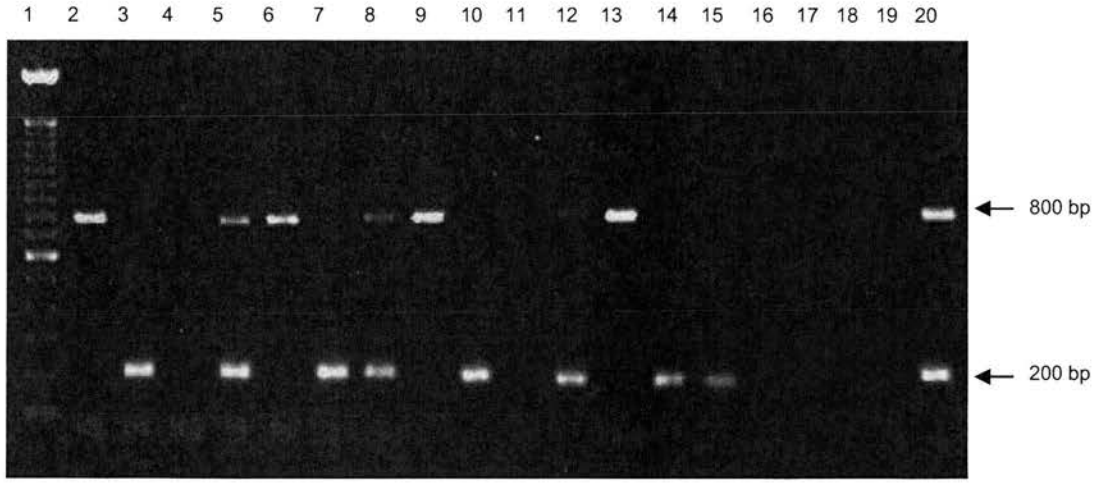

Figure 5.1: Detection of the BoNT/A and BoNT/B toxin genes by PCR. Lane 1, 100bp DNA ladder; lane 2, B303427/1; lane 3, B303427/2; lane 4, B303 534/2; lane 5, B303467; lane 6, B303515/1; lane 7, B303515/2; lane 8, B303853/1; lane 9, B303853/2; lane 10, B303853/3; lane 11, B303853/4; lane 12, B303855/1; lane 13, B303855/2; lane 14, B303855/3; lane 15, B303855/4; lane 16, B303855/5; lane 17, B303855/6; lane 18, B304150; lane 19, B301155a; lane 20, *C. botulinum* type A (MPRL 2509) and *C. botulinum* type B (MPRL 2511).

	PCR results				
Sample	BoNT/A	BoNT/B	BoNT/C	BoNT/D	BoNT/E
B303427/1	+	-	+	-	-
B303427/2	-	+	-	-	-
B303467	+	+	+	-	+
B303515/1	+	-	-	-	-
B303515/2	-	+	-	-	+
B303534/2	-	-	-	-	-
B303853/1	+	+	+	-	-
B303853/2	+	-	+	-	-
B303853/3	-	+	-	-	-
B303853/4	-	-	-	-	+
B303855/1	+	+	-	-	+
B303855/2	+	-	-	-	-
B303855/3	-	+	+	-	-
B303855/4	-	+	-	-	-
B303855/5	-	-	+	-	-
B303855/6	-	-	-	-	+
B304150	-	-	-	-	+
B301155a	-	-	-	-	-
B301155b	-	-	+	-	-
B301155c	-	-	-	-	-

Table 5.2: PCR detection of the BoNT/E gene

Toxin gene profiles of enrichment cultures from the gastrointestinal contents of seabirds.

PCR with the BoNT/E primers produced a 389bp PCR product with DNA from the *C. botulinum* type E strain (MPRL 2512) (Figure 5.2) demonstrating the presence of the BoNT/E gene. Six of the 20 DNA samples taken from the overnight culture of the gastrointestinal contents of seabirds also produced a 389bp PCR product indicating the presence of the BoNT/E gene within the GI contents of those birds (Table 5.2).

PCR detection of the BoNT/C gene

PCR with the Tox384/Tox850 primers produced a 466bp product with DNA from seven of the twenty enrichment cultures indicating the presence of the BoNT/C gene. No additional positive results were seen using the Tox625/Tox 1049 primer pairs or using the nested PCR for the detection of the BoNT/C gene.

PCR detection of the BoNT/D gene

PCR with the DS primers produced a 497bp product from DNA of *C. botulinum* type D demonstrating the presence of the BoNT/D gene. None of the DNA extracts from enrichment cultures were found to contain the gene.

Four of the enrichment cultures were found to contain two neurotoxin genes by PCR (B303427/1B303515/2, B303853/2 and B303855/3). Two of the birds tested positive for three neurotoxin genes (B303853/1 and B303855/1) and one bird, B303467, was found to contain the genes for 4 of the 5 neurotoxins.

5.1.3 Detection of the C2 toxin genes and novyi alpha toxin gene by PCR

PCR with the C2CI primer gave a band of approximately 300bp in seventeen of the twenty enrichment cultures (Fig 5.2) (Table 5.3) indicating the presence of the gene for the enzymatic component of the C2 toxin. Additional bands of varying sizes were observed in some of the samples though it is not clear what these bands represent.

None of the enrichment cultures produced any PCR products with the C2CII primers (Fig 5.2) suggesting that none of the DNA extracts contain the gene for the transport component of the C2 toxin.

PCR with the Nov primers produced a 260bp PCR product with DNA from *C. novyi* type A (MPRL 2530) demonstrating the presence of the gene for the novyi alpha toxin. None of the enrichment cultures were found to contain the novyi alpha toxin gene.

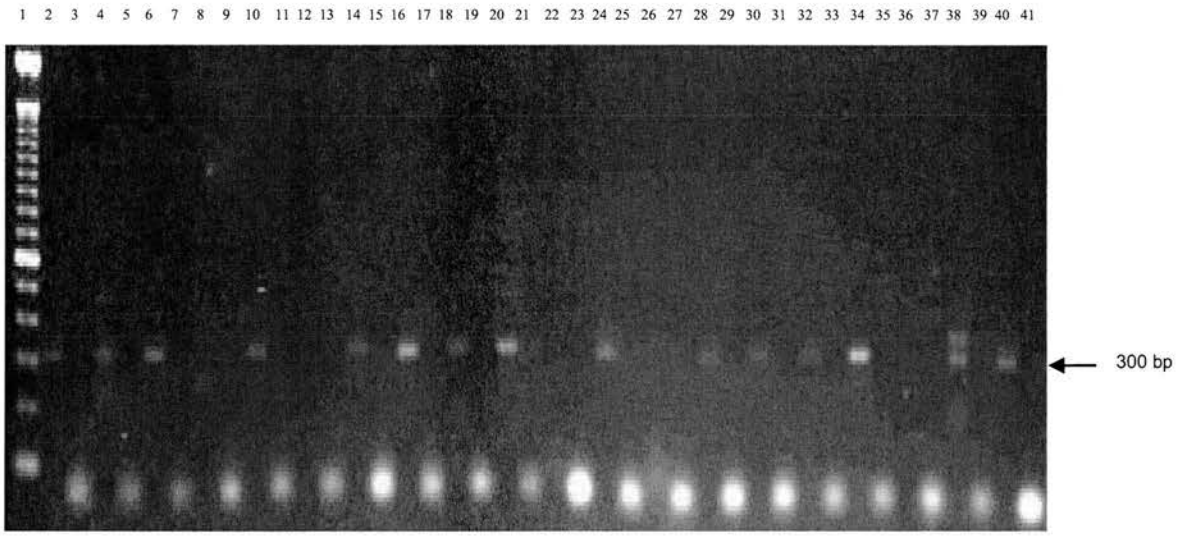

Figure 5.2: PCR detection of the C2 toxin genes. Lane 1, 100bp DNA ladder; lane 2, B303427/1 (C2CI); lane 3, B303427/1 (C2CII); lane 4, B303427/2 (C2CI); lane 5, B303427/2 (C2CII); lane 6, B303467 (C2CI); lane 7, B303467(C2CII); lane 8, B303515/1 (C2CI); lane9, B303515/1 (C2CII); lane 10, B303515/2 (C2CI); lane 11, B303515/2 (C2CII); lane 12, B303534/2 (C2CI); lane 13, B303534/2 (C2CII); lane 14, B303853/1 (C2CI); lane 15, B303853/1 (C2CII); lane 16, B303853/2 (C2CI); lane 17, B303853/2 (C2CII); lane 18, B303853/3 (C2CI); lane 19, B303853/3 (C2CII); lane 20, B303853/4 (C2CI); lane 21, B303853/4 (C2CII); lane 22, B303855/1 (C2CI); lane 23, B303855/1 (C2CII); lane 24, B303855/2 (C2CI); lane 25, B303855/2 (C2CII); lane 26, B303855/3 (C2CI); lane 27, B303855/3 (C2CII); lane 28, B303855/4 (C2CI); lane 29, B303855/4 (C2CII); lane 30, B303855/5 (C2CI); lane 31, B303855/5 (C2CII); lane 32, B303855/6 (C2CI); lane 33, B303855/6 (C2CII); lane 34, B304150 (C2CI); lane 35, B304150 (C2CII); lane 36, B301155a (C2CI); lane 37, B301155a (C2CII); lane 38, B301155b (C2CI); lane 39, B301155b (C2CII); lane 40, B301155c (C2CI); lane 41, B301155c (C2CII).

	PCR results		
Sample	C2CI	C2CII	Novyi α
B303427/1	+	-	-
B303427/2	+	-	-
B303467	+	-	-
B303515/1	+	-	-
B303515/2	+	-	-
B303534/2	+	-	-
B303853/1	+	-	-
B303853/2	+	-	-
B303853/3	+	-	-
B303853/4	+	-	-
B303855/1	-	-	-
B303855/2	-	-	-
B303855/3	+	-	-
B303855/4	+	-	-
B303855/5	+	-	-
B303855/6	+	-	-
B304150	+	-	-
B301155a	-	-	-
B301155b	+	-	-
B301155c	+	-	-

Table 5.3: Group III clostridial toxin gene profile of enrichment cultures from the gastrointestinal contents of seabirds.

5.1.4 Detection of *Clostridium tetani* in soil samples from St. Kilda and Fulmar oil samples.

Isolation of *Clostridium tetani*

All four of the environmental / soil samples collected from St. Kilda were found to contain *C. tetani*-like organisms as demonstrated by the presence of a fine, swarming growth on blood agar. Sub-culture of organisms picked from the leading edge of the swarming colony produce discrete colonies when plated onto 4% agar. Colonies were faintly haemolytic and microscopically resembled *C. tetani* with the characteristic 'drumstick spores' visible in older cultures.

None of the Fulmar oil samples were found to contain organisms resembling *C. tetani* by culture on blood agar. The majority of the Fulmar samples tested contained no viable organisms. The three that were found to contain viable organisms did not appear to be pure samples of Fulmar oil but seemed to be contaminated with stomach contents.

Culture of a laboratory strain of *C. tetani* on blood agar containing a filter paper disc soaked in Fulmar oil produced the typical fine swarming growth. No zone of inhibition was seen around the filter paper and growth of *C. tetani* was observed over the surface of the disc. This suggests that Fulmar oil is not inhibitory to the growth of *C. tetani*.

PCR detection of the *C. tetani* neurotoxin gene

PCR with the Tet primers produced a PCR product of around 1400bp with DNA from *C. tetani* (MPRL 2777) demonstrating the presence of the fragment C of the tetanus neurotoxin. All four of the soil enrichment cultures were found to contain the Tetanus neurotoxin gene. None of the Fulmar oil samples produced any visible PCR product with the Tet primers suggesting that none of them contained the gene for the tetanus neurotoxin.

5.2 Discussion

The direct detection of type BoNT/C in samples B303427/1 and B303467 supports the diagnosis of botulism as the cause of death of these birds. The increase in levels of toxin detected indicates the presence of viable bacteria within the gut contents that are producing toxin. The decrease in the amount of toxin detected following 5 day incubation in samples B303534/2, B303427/4 and B303515/1 suggests that either pre-formed toxin alone is present which is typical of classical food-borne botulism, or that any *C. botulinum* cells present are no longer producing neurotoxin.

Both of the samples that showed an increase in the amount of neurotoxin detected were found to contain the BoNT/C gene by PCR. The gene was not detected in the gut contents of the birds which showed a decrease in the amount of toxin produced supporting the results of the toxin assay. An additional 5 samples were found to contain the BoNT/C gene. The BoNT/D was not detected in any of the samples though the other neurotoxins were found in approximately equal numbers with BoNT/E found in 6 samples, BoNT/A and BoNT/C found in 7 samples and BoNT/B in 8 samples.

Type C and D botulinum are the most common avian forms of the disease with type C being a major cause of mortality in British gulls. The presence of type A neurotoxin and subsequent culture of the organism from the digestive tract of a gull has been documented (Ortiz and Smith, 1994) though this was considered unusual as *C. botulinum* is not a normal component of the gut flora of birds. The detection of

neurotoxin genes in the gut contents of birds in this study indicates the presence of the organism in the gut and not the presence of pre-formed toxin only. It is probable that these organisms had been recently ingested by the birds though the source of them is unknown. Previous studies have suggested that *C. botulinum* type B is the most common strain in British coastal sediments. *C. botulinum* type B has also been shown to be the predominant strain found in seawater fish and coastal sediments with type A and type E also present though in lower numbers (Fach et al, 2002).

It is apparent that *C. botulinum* can be carried by seabirds and the presence of neurotoxin genes in the gut contents suggests that the faeces of seabirds may be a source of disease. Many birds appear to be carrying more than one strain of *C. botulinum.* The majority of birds investigated in this study were herring gulls and this may reflect the feeding behaviour patterns of the birds. The cormorant investigated in this study was only found to contain the BoNT/E gene. All of the samples used in this study had come from birds with suspected or confirmed botulism and therefore the results may not be representative of the seabird population as no healthy controls were available for comparison.

One of the samples of leeches tested positive for the BoNT/C gene however they are unlikely to be the source of the infection and as the bacteria have not been found to be systemic it is thought that this represents contamination of the sample.

The high incidence of the C2 toxin seen in the birds samples is unexpected. The C2 toxin genes have been found to be prevalent in soil and environmental samples and may just represent exposure to soil.

All of the soil samples from St. Kilda were found to contain *C. tetani* by both culture methods and PCR detection of the gene for Fragment C of the neurotoxin. All of the Fulmar oil samples were found to be sterile though it does not appear to be inhibitory to the growth of *C. tetani*.

From this study it seems that the Fulmar oil itself does not appear to have been the source of the tetanus spores that gave such a high incidence of neonatal tetanus on the island. It is likely that the oil was either contaminated during collection or storage or that the umbilicus became contaminated due to a combination of the living conditions and the highly secretive midwifery practices. Fulmar oil is a sticky substance and its presence over the wound site may have increased the risk of contamination as well as acting as a barrier increasing the anaerobic conditions within the wound.

Chapter Six

Discussion

6.1 Isolation and Characterisation of Group III clostridia from equine gastrointestinal contents

This thesis has further investigated the hypothesis that equine grass sickness is caused by toxicoinfection with *C. botulinum* type C and has investigated methods for the isolation and detection of pathogenic clostridia and the characterisation of Group III clostridia by molecular methods.

While the aetiology of equine grass sickness remains unknown, epidemiological studies suggest an infectious aetiology (Wood et al, 1998). The pathology of the disease suggests the involvement of a neurotoxin with the characteristic neuronal degeneration thought to be the primary event in the disease. Toxicoinfection with *C. botulinum* type C has been proposed as the likely cause of grass sickness. The previous detection of Group III-like organisms from the GI tract of horse with grass sickness and the direct detection of BoNT/C in the GI tract combined with the serological studies demonstrating significantly lower levels of antibodies to the surface antigens of Group III clostridia and to BoNT/C demonstrates an association between *C. botulinum* type C and grass sickness (Hunter et al 1999: Hunter and Poxton, 2001).

Traditional culture methods for the isolation of Group III clostridia, in particular *C. botulinum* type C, from the GI tracts of horse with grass sickness were investigated during the course of this thesis. Culture work was hampered by the lack of a selective medium for isolation of the Group III clostridia. A selection of both liquid and solid media were investigated to optimise the recovery of, and toxin production by, *C. botulinum* type C. Overgrowth of Group III clostridia by other organisms in the GI tract remained a problem due to the fastidious nature of these organisms and their slow growth rate. This was further compounded by the increased anaerobic bacterial content of the gastrointestinal contents of horses with grass sickness (Garrett et al 2002). The detection and isolation of Group III clostridia from mixed cultures was further hampered by the potential loss of the typical colony morphology. When grown in a mixed faecal culture, the type strain of *C. botulinum* type C was not consistently found to produce both the lipase effect and lecithinase on egg yolk agar. These characteristics returned upon repeated subculture to obtain a pure culture. It was therefore decided to isolate and further investigate colonies positive for lipase only or lecithinase only from the GI contents of horse with grass sickness as well as those resembling 'typical' Group III clostridia.

Group III-like organisms putatively identified as such by colony morphology and GLC profile were isolated from horses with grass sickness, their co-grazers and control horses, though none of the isolates were found to contain the genes for the species-defining major toxins. It is possible that equine grass sickness may result from the conversion of a non-toxigenic organism in the GI tract by the introduction of the converting phage from an external source. The prevalence of Group III

clostridia in the GI tract of the equine population is not known, though this study suggests that non toxin-producing organisms may be present in the GI tracts of healthy horses with isolates containing the genes for one or both components of the C2 toxin found in the gastrointestinal contents of horses with grass sickness, their co-grazers and control horses. Group III clostridia have also been isolated from soil samples which may provide a reservoir for infection (Nakamura et al 1978).

Due to the problems associated with the culture and isolation of Group III clostridia, it was decided to focus on molecular methods for the detection of toxin genes within the gastrointestinal tract and the characterisation of isolates. PCR detection of the BoNT/C gene yielded multiple products of atypical sizes with multiple bands between 100bp and 800bp present in many of the samples. It is not known if this is due to cross-reactivity with the primers or due to non-specific binding to the primers and subsequent amplification. A Southern blotting methodology was developed and used to detect the BoNT/C gene in whole gut enrichment cultures. A selection of the PCR products obtained from the gastrointestinal enrichment samples were sequenced. Those that matched the sequence of the BoNT/C in a BLAST search were used to obtain a consensus sequence. Two probes for Southern blotting were then ordered based on these sequences.

In this preliminary study, the BoNT/C gene was detected in four of the seven horses with confirmed grass sickness and in none of the control animals by Southern blotting. The BoNT/C gene was detected in enrichment cultures of two or more sites in all four animals. This is consistent with the pattern of neuronal degeneration seen

along the length of the gastrointestinal tract in horses with acute grass sickness (Scholes et al. 1993) though it is not known if the toxin was produced at these sites. The detection of the toxin gene itself within the gastrointestinal tract of horse with acute grass sickness supports the hypothesis that grass sickness is caused by a toxicoinfection with *C. botulinum* type C with locally produced toxin causing the neuropathology that is characteristic of the disease.

The BoNT/C gene was detected in the ileum of only one of the horses tested. The neuronal damage seen in grass sickness is usually severe and extensive in the ileum (Scholes et al. 1993) and it has been proposed that, in acute cases, the animals are exposed to large amounts of toxin in the ileum and jejunum in a short period of time. It would therefore be expected that the BoNT/C gene would be present in the ileal contents of all of the animals with confirmed acute grass sickness. The absence may reflect the small sample size used in this study or the degradation of the toxin gene prior to the euthanasia of the animal or during the enrichment culture. The detection of IgA to BoNT/C in the GI tracts of horses with grass sickness (Nunn et al, 2007) suggests exposure to the toxin prior to the onset of symptoms in horses with acute grass sickness. It is possible that the toxigenic organisms or free bacteriophages in the GI tract are no longer present in the gut at the time of sampling.

6.2 Molecular characterisation and detection of toxin genes of Group III clostridia

The detection of isolates from the gastrointestinal contents of horses containing genes for one or both of the components of the C2 toxin but none of the defining major toxins led to a study on the distribution of toxin genes in a selection of laboratory isolates of *C. botulinum* types C and D and *C. novyi* type A.

The C2 toxin is documented as being produced by the majority of *C. botulinum* type C strains and by some type D strains (Fujii et al, 1996; Kimura et al, 1998). Its presence in *C. novyi* type A has not been reported. The C2 toxin is a binary ADP-ribosylating toxin composed of an enzymatic portion (C2CI) and a binding portion (C2CII).

This study found a high incidence of the gene for the C2CI enzymatic component in isolates from a variety of sources, both clinical and environmental but a much lower incidence of the gene for the C2CII binding component. Two of the three *C. botulinum* type C strains and the *C. botulinum* type D strain tested were found to contain the genes for both components of the C2 toxin as expected based on the current literature. One of the three *C. botulinum* type C strains was found to contain the gene for the C2CI only. It is not clear if the C2CII gene has been lost in this strain or if sequence heterogeneity within the C2CII gene is responsible for a mis-match with the primers. The primers used to identify the C2CII gene had been shown to amplify DNA from all three groups identified based upon the molecular weight of the C2 toxin produced (Kimura et al, 1998) though this was based upon a small

sample size so it is possible that there are further sequence variations within the C2CII gene.

Of the 40 strains identified as *C. novyi* type A in the laboratory culture collection, only 22 were found to contain the *C. novyi* α toxin gene by PCR and 6 strains were found to contain none of the toxin genes investigated. All of the clinical isolates obtained from the outbreak of *C. novyi* wound infections in injecting drug users were found to contain the *C. novyi* α toxin gene which is consistent with the pathology of the disease.

Though the C2 toxin has not been previously reported in *C. novyi* type A, this study found that 2 of the 40 strains tested contained the genes for both components of the C2 toxin and 30 of the 40 strains tested contained the genes for one of the components of the C2 toxin with the enzymatic component found in the majority of strains. It is not known if these genes are being expressed by the bacteria. There is no evidence that the C2CI alone has a role in pathogenesis though it is possible that it may be able to enter damaged cells independent of the C2CII binding component and add to the pathology of disease. The C2 toxin has been implicated in disease in botulism in broiler chickens and horses (Ohishi and DasGupta, 1987; Kinde et al, 1991).

This further demonstrates the close relationship between these organisms and complicates the identification of the Group III clostridia to species level and the characterisation of isolates in which the major toxin gene has been lost. *C. botulinum*

type C and D are considered to be separate species based on a 1% sequence divergence of the 16S rRNA gene (Collins and East, 1998) and *C. novyi* is considered to be a separate species based upon a 2% sequence divergence within the 16S rRNA gene. The Group III clostridia can only be identified to species level by the detection of the major toxin. This thesis has highlighted the problems in identifying bacteria based upon the detection of a toxin. The species-defining toxins are carried on pseudolysogenic bacteriophages. The phage-host relationship is unstable and the phages are readily lost upon sub-culture. In the absence of the major toxin, these bacterial strains are indistinguishable from each other by basic culture and PCR methodologies. Given the high degree of sequence heterogeneity seen in clostridial genes, it is questionable whether a 1-2% sequence divergence is enough to be able to class these organisms as separate species, particularly when the bacteriophages that carry the species-defining toxins are potentially able to infect all three species of Group III clostridia, changing the identity of the cell they infect.

It has been proposed that there exists a common, non-toxigenic, strain and that its pathogenicity is determined by the toxin it produces (Eklund and Poysky, 1974). The results obtained in this thesis support the hypothesis that the Group III clostridia cannot be defined to a species level without the detection of the major toxin and that there is not sufficient difference between *C. botulinum* types C and D and *C. novyi* type A for them to be considered separate species in the absence of the converting bacteriophages.

6.3 Investigation into the prevalence of toxigenic clostridia in seabirds

This thesis also applied the techniques developed for the detection of toxin genes in clostridia to investigate the distribution of clostridial neurotoxin genes in seabirds. Outbreaks of botulism occur frequently in birds and though they are susceptible to all types of botulinum toxin, type C botulism is responsible for the majority of cases (Lamanna, 1987). This study found detectable levels BoNT/C in 4 of the 6 samples of gastrointestinal contents of seabirds found dead with suspected botulism which is consistent with the published observations. Two of the samples showed an increase in the amount of toxin detected over the 5 day incubation period suggesting the presence of viable bacteria within the gut contents that are actively producing toxin. This was confirmed by the PCR detection of the BoNT/C gene. In this study 8 of the 20 samples investigated were found to contain the gene for BoNT/B, 7 samples were found to contain the gene for BoNT/C and no samples were found to contain the gene for BoNT/D. Spores of *C. botulinum* types B, C and D were found in 63% of landfill sites (Ortiz and Smith 1994) which is consistent with the results obtained here for BoNT/B and BoNT/C.

This study detected the gene for BoNT/A in 7 of the 40 samples tested and the gene for BoNT/E in 6 of the samples. The presence of type A neurotoxin from the digestive tract of a gull has been previously reported but was considered unusual (Ortiz and Smith, 1994). This study suggests that the carriage of *C. botulinum* by seabirds may be more common than previously thought though all of the samples

used in this study were from birds with suspected or confirmed botulism and so may not be representative of the wider population.

The detection of a range of clostridial neurotoxin genes in the GI contents of seabirds confirms the presence of toxin-producing organisms in the GI tract of birds. This suggests that seabirds are able to act as a source of botulism and may be responsible for the contamination of environmental sites with spores of *C. botulinum*. The source of these organisms is not clear though *C. botulinum* type B has been shown to be the predominant strain in seawater fish and coastal sediments with *C. botulinum* types A and E also present (Fach et al, 2002).

Neonatal tetanus remains the most common form of the disease worldwide and was thought to be a contributory factor in the evacuation of the island of St Kilda in 1930, due largely to the decline in the population. This high incidence of the disease was thought to be due to the practice of anointing the umbilicus of newborn babies with oil collected from the Fulmar.

This study suggests that Fulmar oil is not a source of tetanus. All of the Fulmar oil samples tested in this study were found to be sterile, though the oil itself was not found to be inhibitory to the growth of *C. tetani.* The gene for the tetanus toxin was found in all soil samples collected from St. Kilda as expected given the worldwide distribution of the organism in soil. It is possible that the Fulmar oil was contaminated with this soil-borne organism during collection or storage. We

conclude that the general living conditions were the more likely cause of the disease though the fulmar oil may have contributed to the development of an anaerobic environment within the wound site or simply be preventing the drying out and healing of the area.

6.4 Conclusions and Further work

The aetiology of equine grass sickness remains unknown though epidemiological studies suggest that grass sickness may have an infectious aetiology. The pathology of the disease suggests a toxic aetiology. Toxicoinfection with *C. botulinum* type C has been suggested as a cause of the disease and one which fits both of these criteria. The detection of BoNT/C and BoNT/C-producing organisms in the gastrointestinal tract of horses with grass sickness demonstrated a strong association between *C. botulinum* type C and the disease. Serological studies have provided further evidence of a link between *C. botulinum* type C and grass sickness. Horses with grass sickness were found to have significantly lower levels of IgG to both surface antigens and BoNT/C (Hunter and Poxton, 2001). This may be due to the *in vivo* neutralisation of the organism and toxin resulting in a lower level of IgG available for detection or it may be that lower levels of IgG are a risk factor for the development of the disease with low antibody levels to the surface antigens allowing the proliferation of the bacteria within the gastrointestinal tract. The detection of IgA to BoNT/C in horses with grass sickness confirms recent exposure to the toxin, and in acute cases this may be prior to the onset of gastrointestinal stasis.

The detection of the BoNT/C gene in a variety of sites along the gastrointestinal tract of horses with confirmed grass sickness further demonstrates a link between *C. botulinum* type C and grass sickness and provides evidence of the potential ability for toxin production within the gastrointestinal tract consistent with a toxicoinfection. The low level of detection of the BoNT/C gene in the ileal contents may further support the serological data suggesting exposure to the toxin prior to the onset of clinical symptoms and the theory that acute grass sickness is caused by exposure to high levels of toxin over a short period of time.

Further work is required to confirm the identity of the genes identified as BoNT/C by Southern blotting in this study by sequencing. Additionally RT-PCR to investigate toxin production within the gastrointestinal tract may provide further evidence of a toxicoinfection. At present the prevalence of Group III clostridia within the gastrointestinal tract of the equine population is not known. This study suggests that non-toxigenic Group III organisms may be present in healthy horses as well as those with grass sickness but sequencing of the C2 toxin genes identified by PCR would be required to confirm this. The source of *C. botulinum* should be established. If, as has been suggested, grass sickness results from the conversion of a non-toxigenic organism within the gastrointestinal tract by the introduction of the converting phage then the source of the phage needs to be established. Investigation of pasture for the presence of Group III clostridia or the converting phages should determine whether there is an environmental reservoir. The molecular methods developed in this thesis form the basis for the identification of Group III clostridia and the major toxin genes without the need to culture viable, toxigenic organisms.

This thesis has also highlighted the difficulties in identifying the Group III clostridia to species level when the species-defining toxin is not present. Further sequencing of a large number of Group III clostridia may determine whether they are different enough to be considered separate species in the absence of the major toxin. Further investigation into the carriage of the C2 toxin genes by these organisms is needed to determine how widespread the carriage of this toxin is. In particular, investigation of the primers used to detect the C2CII gene is required to eliminate the possibility of false negatives due to sequence heterogeneity. Confirmation of PCR products by sequencing is needed, particularly in strains carrying the gene for only one component of the toxin. RT-PCR would determine whether the genes are being expressed.

References

Aktories, K., M. Barmann, I. Ohishi, S. Tsuyama, K. H. Jakobs and E. Habermann (1986).Botulinum-C2 Toxin Adp-Ribosylates Actin *Nature* **322**: 390-392.

Aktories, K, and A. Wegner. (1989). ADP-ribosylation of actin by clostridial toxins. *Journal of Cell Biology*, **109**: 1385-1387.

Aktories, K. and A. Wegner (1992).Mechanisms of the Cytopathic Action of Actin-Adp-Ribosylating Toxins *Molecular Microbiology* **6**: 2905-2908.

Arnon, S. S. (1980).Honey, Infant Botulism and the Sudden Infant Death Syndrome *Western Journal of Medicine* **132**: 58-59.

Ball, D. W., R. L. Vantassell, M. D. Roberts, P. E. Hahn, D. M. Lyerly and T. D. Wilkins (1993).Purification and Characterization of Alpha-Toxin Produced by Clostridium-Novyi Type-A *Infection and Immunity* **61**: 2912-2918.

Barlow, R. M. (1969).Neuropathological Observations in Grass Sickness of Horses *Journal of Comparative Pathology* **79**: 407-&.

Barth, H., D. Blocker, J. Behlke, W. Bergsma-Schutter, A. Brisson, R. Benz and K. Aktories (2000).Cellular uptake of Clostridium botulinum C2 toxin requires oligomerization and acidification *Journal of Biological Chemistry* **275**: 18704-18711.

Barth, H., F. Hofmann, C. Olenik, I. Just and K. Aktories (1998).The N-terminal part of the enzyme component (C2I) of the binary Clostridium botulinum C2 toxin

interacts with the binding component C2II and functions as a carrier system for a Rho ADP- ribosylating C3-like fusion toxin *Infection and Immunity* **66**: 1364-1369.

Barth, H., J. C. Preiss, F. Hofmann and K. Aktories (1998).Characterization of the catalytic site of the ADP- ribosyltransferase Clostridium botulinum C2 toxin by site-directed mutagenesis **273**: 29506-29511.

Bishop, A. L. and A. Hall (2000).Rho GTPases and their effector proteins *Biochemical Journal* **348**: 241-255.

Bizzini, B. (1979).Tetanus Toxin *Microbiological Reviews* **43**: 224-240.

Blocker, D., H. Barth, E. Maier, R. Benz, J. T. Barbieri and K. Aktories (2000).The C terminus of component C2II of Clostridium botulinum C2 toxin is essential for receptor binding *Infection and Immunity* **68**: 4566-4573.

Brown, R., J. G. Collee, and I. R. Poxton. (1996). Bacteroides, Fusobacterium and other Gram-negative anaerobic rods; anaerobic cocci; identification of anaerobes. *In* J. G. Collee, A. G. Fraser, B. P. Marmion, and A. Simmons (ed.), Mackie and McCartney Practical Medical Microbiology 14th edition. Churchill Livingston: Edinburgh. 501-519.

Busch, C. and K. Aktories (2000).Microbial toxins and the glycosylation of Rho family GTPases *Current Opinion in Structural Biology* **10**: 528-535.

Busch, C., F. Hofmann, R. Gerhard and K. Aktories (2000).Involvement of a conserved tryptophan residue in the UDP-glucose binding of large clostridial cytotoxin glycosyltransferases *Journal of Biological Chemistry* **275**: 13228-13234.

Chia, J. K., J. B. Clark, C. A. Ryan and M. Pollack (1986).Botulism in an Adult Associated with Foodborne Intestinal Infection with Clostridium-Botulinum *New England Journal of Medicine* **315**: 239-241.

Coffield, J. A., N. Bakry, R. D. Zhang, J. Carlson, L. G. Gomella and L. L. Simpson (1997).In vitro characterization of botulinum toxin types A, C and D action on human tissues: Combined electrophysiologic, pharmacologic and molecular biologic approaches *Journal of Pharmacology and Experimental Therapeutics* **280**: 1489-1498.

Collins, M. D. and A. K. East (1998).Phylogeny and taxonomy of the food-borne pathogen Clostridium botulinum and its neurotoxins *Journal of Applied Microbiology* **84**: 5-17.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acid Research*, **16**: 10881-10890.

Deinhardt, K., O. Berninghausen, H. J. Willison, C. R. Hopkins and G. Schiavo (2006).Tetanus toxin is internalized by a sequential clathrin-dependent mechanism initiated within lipid microdomains and independent of epsin 1 *Journal of Cell Biology* **174**: 459-471.

Delamballerie, X., C. Zandotti, C. Vignoli, C. Bollet and P. Demicco (1992).A One-Step Microbial Dna Extraction Method Using Chelex-100 Suitable For Gene Amplification *Research in Microbiology* **143**: 785-790.

Donovan, J. J. and J. L. Middlebrook (1986).Ion-Conducting Channels Produced by Botulinum Toxin in Planar Lipid-Membranes *Biochemistry* **25**: 2872-2876.

Doxey, D. L., J. S. Gilmour and E. M. Milne (1991).The Relationship between Meteorological Features and Equine Grass Sickness (Dysautonomia) *Equine Veterinary Journal* **23**: 370-373.

Doxey, D. L., E. M. Milne, J. S. Gilmour and D. M. Pogson (1991).Clinical and Biochemical Features of Grass Sickness (Equine Dysautonomia) *Equine Veterinary Journal* **23**: 360-364.

Doxey, D. L., D. M. Pogson, E. M. Milne, J. S. Gilmour and H. K. Chisholm (1992).Clinical Equine Dysautonomia and Autonomic Neuron Damage *Research in Veterinary Science* **53**: 106-109.

Doxey, D. L., E. M. Milne, M. P. Woodman, J. S. Gilmour and H. K. Chisholm (1995).Small-Intestine and Small Colon Neuropathy in Equine Dysautonomia (Grass Sickness) *Veterinary Research Communications* **19**: 529-543.

Doxey, D. L., D. M. Pogson, E. M. Milne, J. S. Gilmour and H. K. Chisholm (1992).Clinical Equine Dysautonomia and Autonomic Neuron Damage *Research in Veterinary Science* **53**: 106-109.

Eckhardt, M., H. Barth, D. Blocker and K. Aktories (2000).Binding of Clostridium botulinum C2 toxin to asparagine-linked complex and hybrid carbohydrates *Journal of Biological Chemistry* **275**: 2328-2334.

Eklund, M. W. and F. T. Poysky (1974).Interconversion of Type-C and D Strains of Clostridium-Botulinum by Specific Bacteriophages *Applied Microbiology* **27**: 251-258.

Eklund, M. W., F. T. Poysky, J. A. Meyers and G. A. Pelroy (1974).Interspecies Conversion of Clostridium-Botulinum Type-C to Clostridium-Novyi Type-a by Bacteriophage *Science* **186**: 456-458.

Eklund, M. W., F. T. Poysky, M. E. Peterson and J. A. Meyers (1976).Relationship of Bacteriophages to Alpha-Toxin Production in Clostridium-Novyi Type-a and Type-B *Infection and Immunity* **14**: 793-803.

Eklund, M. W., F. T. Poysky and S. M. Reed (1972).Bacteriophage and Toxigenicity of Clostridium-Botulinum Type-D *Nature-New Biology* **235**: 16-&.

Eklund, M. W., F. T. Poysky, S. M. Reed and C. A. Smith (1971).Bacteriophage and Toxigenicity of Clostridium Botulinum Type-C *Science* **172**: 480-&.

Eklund, M. W., F. Poysky, K. Oguma, H. Iida, and K. Inoue (1987). Relationship of bacteriophages to toxin and hemagglutinin production and its significance in avian botulism outbreaks *In* M. W. Eklund and V. R. Dowell, Jr (ed.), Avian botulism: an international perspective. Charles C. Thomas, Springfield Ill. 191-222

Eklund, M. W., F. T. Poysky, and W. H. Habig. (1989). Bacteriophages and plasmids in *Clostridium botulinum* and *Clostridium tetani* and their relationship to production of toxins *In* L. L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press: London, 25-51.

Eleopra, R., V. Tugnoli, O. Rossetto, C. Montecucco and D. DeGrandis (1997).Botulinum neurotoxin serotype C: A novel effective botulinum toxin therapy in human *Neuroscience Letters* **224**: 91-94.

Fach, P., S. Perelle, F. Dilasser, J. Grout, C. Dargaignaratz, L. Botella, J. M. Gourreau, F. Carlin, M. R. Popoff and V. Broussolle (2002).Detection by PCR-enzyme-linked immunosorbent assay of Clostridium botulinum in fish and environmental samples from a coastal area in northern France *Applied and Environmental Microbiology* **68**: 5870-5876.

Fujii, N., T. Kubota, S. Shirakawa, K. Kimura, I. Ohishi, K. Moriishi, E. Isogai and H. Isogai (1996).Characterization of component-I gene of botulinum C2 toxin and PCR detection of its gene in clostridial species *Biochemical and Biophysical Research Communications* **220**: 353-359.

Fujinaga, Y., K. Inoue, S. Watanabe, K. Yokota, Y. Hirai, E. Nagamachi and K. Oguma (1997).The haemagglutinin of Clostridium botulinum type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of the toxin **143**: 3841-3847.

Garrett, L. A., R. Brown and I. R. Poxton (2002).A comparative study of the intestinal microbiota of healthy horses and those suffering from equine grass sickness *Veterinary Microbiology* **87**: 81-88.

Gilmour, J. S. (1973).Observations on Neuronal Changes in Grass Sickness of Horses *Research in Veterinary Science* **15**: 197-200.

Gilmour, J. S. (1987).Equine Dysautonomia - Epidemiology and Pathology *Journal of Small Animal Practice* **28**: 373-380.

Gilmour, J. S. and G. M. Jolly (1974).Some Aspects of Epidemiology of Equine Grass Sickness *Veterinary Record* **95**: 77-&.

Griffiths, I. R., S. Smith, D. L. Doxey, K. Whitwell and S. Love (1994).Evidence That the Agent of Equine Grass Sickness May Reach Neurons by Retrograde Axonal-Transport *Veterinary Record* **135**: 520-523.

Haagsma, J. (1987), Laboratory investigation of botulism in wild birds. *In* M. W. Eklund and V. R. Dowell, Jr (ed.), Avian botulism: an international perspective. Charles C. Thomas, Springfield Ill. 282-293.

Hall, A. (1998).Rho GTPases and the actin cytoskeleton *Science* **279**: 509-514.

Hall, J. D., L. M. McCroskey, B. J. Pincomb and C. L. Hatheway (1985).Isolation of an Organism Resembling Clostridium-Barati Which Produces Type-F Botulinal Toxin from an Infant with Botulism *Journal of Clinical Microbiology* **21**: 654-655.

Halpern, J. L., W. H. Habig, E. A. Neale and S. Stibitz (1990).Cloning and Expression of Functional Fragment-C of Tetanus Toxin *Infection and Immunity,* ***58***: 1004-1009.

Hariharan, H. and W. R. Mitchell (1976).Observations on bacteriophages of Clostridium botulinum type C isolates from different sources and the role of certain phages in toxigenicity *Appl Environ Microbiol* **32**: 145-158.

Hatheway, C. L., (1989) Bacterial sources of clostridial neurotoxins. *In* L. L. simpson (ed.), Botulinum neurotoxin and tetanus neurotoxin. Academic Press. 4-24.

Hatheway, C. L. (1990).Toxigenic Clostridia *Clinical Microbiology Reviews* **3**: 66-98.

Hatheway, C. L. and L. M. McCroskey (1987).Examination of Feces and Serum for Diagnosis of Infant Botulism in 336 Patients *Journal of Clinical Microbiology* **25**: 2334-2338.

Hauser, D., M. Gibert, J. C. Marvaud, M. W. Eklund and M. R. Popoff (1995).Botulinal Neurotoxin C1 Complex Genes, Clostridial Neurotoxin Homology and Genetic Transfer in Clostridium-Botulinum *Toxicon* **33**: 515-526.

Hayashi, T., H. McMahon, S. Yamasaki, T. Binz, Y. Hata, T. C. Sudhof and H. Niemann (1994).Synaptic Vesicle Membrane-Fusion Complex - Action of Clostridial Neurotoxins on Assembly *Embo Journal* **13**: 5051-5061.

Helting, T. B., S. Parschat and H. Engelhardt (1979).Structure of Tetanus Toxin - Demonstration and Separation of a Specific Enzyme Converting Intracellular Tetanus Toxin to the Extracellular Form *Journal of Biological Chemistry* **254**: 728-733.

Hofmann, F., A. Herrmann, E. Habermann and C. Voneichelstreiber (1995).Sequencing and Analysis of the Gene Encoding the Alpha-Toxin of Clostridium-Novyi Proves Its Homology to Toxin-a and Toxin-B of Clostridium-Difficile *Molecular & General Genetics* **247**: 670-679.

Hunter, L. C., J. K. Miller and I. R. Poxton (1999).The association of Clostridium botulinum type C with equine grass sickness: a toxicoinfection? *Equine Vet J* **31**: 492-499.

Hunter, L. C. and I. R. Poxton (2001).Systemic antibodies to Clostridium botulinum type C: do they protect horses from grass sickness (dysautonomia)? *Equine Veterinary Journal* **33**: 547-553.

Hutson, R. A., D. E. Thompson and M. D. Collins (1993).Genetic Interrelationships of Saccharolytic Clostridium- Botulinum Type-B, Type-E and Type-F and Related Clostridia as Revealed by Small-Subunit Ribosomal-Rna Gene-Sequences **108**: 103-110.

Hutson, R. A., D. E. Thompson, P. A. Lawson, R. P. Schockenitturino, E. C. Bottger and M. D. Collins (1993).Genetic Interrelationships of Proteolytic Clostridium-Botulinum Type-a, Type-B, and Type-F and Other Members of the Clostridium-Botulinum Complex as Revealed by Small-Subunit Ribosomal-Rna Gene-Sequences *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **64**: 273-283.

Inoue, K., Y. Fujinaga, K. Honke, K. Yokota, T. Ikeda, T. Ohyama, K. Takeshi, T. Watanabe and K. Oguma (1999).Characterization of haemagglutinin activity of Clostridium botulinum type C and D 16S toxins, and one subcomponent of haemagglutinin (HA1) *Microbiology-Uk* **145**: 2533.

Jenson, W. I., and J. I. Price. (1987). The global importance of type C botulism in wild birds. *In* M. W. Eklund and V. R. Dowell (ed.), Avian Botulism: An International Perspective. Charles C. Thomas, Springfiled Illinois. 33-54.

Kimura, K., T. Kubota, I. Ohishi, E. Isogai, H. Isogai and N. Fujii (1998).The gene for component-II of botulinum C2 toxin *Veterinary Microbiology* **62**: 27-34.

Kinde, H., R. L. Bettey, A. Ardans, F. D. Galey, B. M. Daft, R. L. Walker, M. W. Eklund and J. W. Byrd (1991).Clostridium-Botulinum Type-C Intoxication Associated with Consumption of Processed Alfalfa Hay Cubes in Horses *Journal of the American Veterinary Medical Association* **199**: 742-746.

Kozaki, S., G. Sakaguchi, M. Nishimura, M. Iwamori and Y. Nagai (1984).Inhibitory Effect of Ganglioside-Gt1b on the Activities of Clostridium-Botulinum Toxins *Fems Microbiology Letters* **21**: 219-223.

Kurokawa, Y., K. Oguma, N. Yokosawa, B. Syuto, R. Fukatsu and I. Yamashita (1987).Binding and Cytotoxic Effects of Clostridium-Botulinum Type-a, Type-C1 and Type-E Toxins in Primary Neuron Cultures from Fetal Mouse Brains *Journal of General Microbiology* **133**: 2647-2657.

Lamanna, C. (1987). The scope of the avian botulism problem. *In* M. W. Eklund and V. R. Dowell, Jr (ed.), Avian botulism: an international perspective. Charles C. Thomas, Springfield Ill. 5-11

Lee, W. H. and H. Riemann (1970).Correlation of Toxic and Non-Toxic Strains of Clostridium-Botulinum by DNA Composition and Homology *Journal of General Microbiology* **60**: 117-&.

Lindstrom, M., R. Keto, A. Markkula, M. Nevas, S. Hielm and H. Korkeala (2001).Multiplex PCR assay for detection and identification of Clostridium botulinum types A, B, E, and F in food and fecal material *Applied and Environmental Microbiology* **67**: 5694-5699.

Macdonald, K. L., M. L. Cohen and P. A. Blake (1986).The Changing Epidemiology of Adult Botulism in the United-States *American Journal of Epidemiology* **124**: 794-799.

Maclennan, J. D. (1962).Histotoxic Clostridial Infections of Man *Bacteriological Reviews* **26**: 177-&.

Maksymowych, A. B. and L. L. Simpson (1998).Binding and transcytosis of botulinum neurotoxin by polarized human colon carcinoma cells *Journal of Biological Chemistry* **273**: 21950-21957.

McCarthy, H. E., N. P. French, G. B. Edwards, K. Miller and C. J. Proudman (2004a).Why are certain premises at increased risk of equine grass sickness? A matched case-control study *Equine Veterinary Journal* **36**: 130-134.

McCarthy, H. E., N. P. French, G. B. Edwards, I. R. Poxton, D. F. Kelly, C. E. Payne-Johnson, K. Miller and C. J. Proudman (2004b).Equine grass sickness is associated with low antibody levels to Clostridium botulinum: a matched case-control study *Equine Veterinary Journal* **36**: 123-129.

McCroskey, L. M. and C. L. Hatheway (1988).Laboratory Findings in 4 Cases of Adult Botulism Suggest Colonization of the Intestinal-Tract *Journal of Clinical Microbiology* **26**: 1052-1054.

McCroskey, L. M., C. L. Hatheway, L. Fenicia, B. Pasolini and P. Aureli (1986).Characterization of an Organism That Produces Type-E Botulinal Toxin but Which Resembles Clostridium-Butyricum from the Feces of an Infant with Type-E Botulism *Journal of Clinical Microbiology* **23**: 201-202.

McGorum, B. C., S. C. Fry, G. Wallace, K. Coenen, J. Robb, G. Williamson and O. I. Aruoma (2000).Properties of herbage in relation to equine dysautonomia: Biochemical composition and antioxidant and prooxidant actions *Journal of Agricultural and Food Chemistry* **48**: 2346-2352.

McGuigan, C. C., G. M. Penrice, L. Gruer, S. Ahmed, D. Goldberg, M. Black, J. E. Salmon and J. Hood (2002).Lethal outbreak of infection with Clostridium novyi type A and other spore-forming organisms in Scottish injecting drug users **51**: 971-977.

McLauchlin, J., J. E. Salmon, S. Ahmed, J. S. Brazier, M. M. Brett, R. C. George and J. Hood (2002).Amplified fragment length polymorphism (AFLP) analysis of Clostridium novyi, C-perfringens and Bacillus cereus isolated from injecting drug users during 2000 **51**: 990-1000.

McLoughlin, M. F., S. G. McIlroy and S. D. Neill (1988).A Major Outbreak of Botulism in Cattle Being Fed Ensiled Poultry Litter *Veterinary Record* **122**: 579-581.

Milne, E. M., M. P. Woodman and D. L. Doxey (1994).Use of Clinical Measurements to Predict the Outcome in Chronic Cases of Grass Sickness (Equine Dysautonomia) *Veterinary Record* **134**: 438-440.

Mitchell, W. R., and S. Rosendal (1987). Type C botulism: the agent, host susceptibility, and predisposing factors. *In* M. W. Eklund and V. R. Dowell, Jr (ed.), Avian botulism: an international perspective. Charles C. Thomas, Springfield Ill. 55-71.

Montecucco, C. (1986).How Do Tetanus and Botulinum Toxins Bind to Neuronal Membranes *Trends in Biochemical Sciences* **11**: 314-317.

Montecucco, C., E. Papini and G. Schiavo (1994).Bacterial Protein Toxins Penetrate Cells Via a 4-Step Mechanism *Febs Letters* **346**: 92-98.

Montecucco, C. and G. Schiavo (1994).Mechanism of Action of Tetanus and Botulinum Neurotoxins *Molecular Microbiology* **13**: 1-8.

Moriishi, K., M. Koura, N. Abe, N. Fujii, Y. Fujinaga, K. Inoue and K. Ogumad (1996).Mosaic structures of neurotoxins produced from Clostridium botulinum types C and D organisms *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1307**: 123-126.

Moriishi, K., B. Syuto, M. Saito, K. Oguma, N. Fujii, N. Abe and M. Naiki (1993).2 Different Types of Adp-Ribosyltransferase C3 from Clostridium-Botulinum Type D Lysogenized Organisms *Infection and Immunity* **61**: 5309-5314.

Morris, N. P., E. Consiglio, L. D. Kohn, W. H. Habig, M. C. Hardegree and T. B. Helting (1980).Interaction of Fragment-B and Fragment-C of Tetanus Toxin with Neural and Thyroid Membranes and with Gangliosides *Journal of Biological Chemistry* **255**: 6071-6076.

Murray, A., D. F. Cottrell and M. P. Woodman (1994).Cholinergic Activity of Intestinal Muscle in-Vitro Taken from Horses with and without Equine Grass Sickness *Veterinary Research Communications* **18**: 199-207.

Nakamura, S., I. Kimura, K. Yamakawa and S. Nishida (1983).Taxonomic Relationships among Clostridium-Novyi Type-a and Type-B, Clostridium-Hemolyticum and Clostridium-Botulinum Type-C *Journal of General Microbiology* **129**: 1473-1479.

Nakamura, S., T. Serikawa, K. Yamakawa, S. Nishida, S. Kozaki and G. Sakaguchi (1978).Sporulation and C2 Toxin Production by Clostridium-Botulinum Type-C Strains Producing No C1 Toxin *Microbiology and Immunology* **22**: 591-596.

Norgauer, J., E. Kownatzki, R. Seifert and K. Aktories (1988).Botulinum C2-Toxin Adp-Ribosylates Actin and Enhances O-2- Production and Secretion but Inhibits Migration of Activated Human-Neutrophils *Journal of Clinical Investigation* **82**: 1376-1382.

Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig and H. Backhaus (1996).Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis **178**: 5636-5643.

Nunn, F. G., R. S. Pirie, B. McGorum, U. Wernery, and I. R. Poxton (2007). Prelimary study of mucosal IgA in the equine small intestine: Specific IgA in cases of acute grass sickness and controls *Equine Veterinary Journal* **39:**457-460.

Obel, A. L. (1955). Studies on grass disease. The morphological picture with special reference to the vegetative nervous system. *Journal of Comparative Pathology*, **65**: 334-346.

Oguma, K., Y. Fujinaga and K. Inoue (1995).Structure and Function of Clostridium-Botulinum Toxins *Microbiology and Immunology* **39**: 161-168.

Oguma, K. and H. Iida (1979).High and Low Toxin Production by a Non-Toxigenic Strain of Clostridium-Botulinum Type-C Following Infection with Type-C Phages of Different Passage History *Journal of General Microbiology* **112**: 203-206.

Oguma, K., H. Iida, M. Shiozaki and K. Inoue (1976).Antigenicity of Converting Phages Obtained from Clostridium-Botulinum Type-C and Type-D *Infection and Immunity* **13**: 855-860.

Ohishi, I. (1983).Lethal and Vascular-Permeability Activities of Botulinum C-2 Toxin Induced by Separate Injections of the 2 Toxin Components *Infection and Immunity* **40**: 336-339.

Ohishi, I. and Y. Odagiri (1984).Histopathological Effect of Botulinum-C2 Toxin on Mouse Intestines *Infection and Immunity* **43**: 54-58.

Ohishi, I., and B. R. Dasgupta (1987). Molecular structure and biological activities of *Clostridium botulinum* C_2 toxin. *In* M. W. Eklund and V. R. Dowell, Jr (ed.), Avian botulism: an international perspective. Charles C. Thomas, Publisher, Springfield Illinois. 223-247

Ohishi, I. and G. Sakaguchi (1980).Oral Toxicities of Clostridium-Botulinum Type-C and Type-D Toxins of Different Molecular Sizes *Infection and Immunity* **28**: 303-309.

Ortiz, N. E. and G. R. Smith (1994).Landfill Sites, Botulism and Gulls *Epidemiology and Infection* **112**: 385-391.

Ortolani, E. L., L. A. B. Brito, C. S. Mori, U. Schalch, J. Pacheco and L. Baldacci (1997).Botulism outbreak associated with poultry litter consumption in three Brazilian cattle herds *Veterinary and Human Toxicology* **39**: 89-92.

OsenSand, A., J. K. Staple, E. Naldi, G. Schiavo, O. Rossetto, S. Petitpierre, A. Malgaroli, C. Montecucco and S. Catsicas (1996).Common and distinct fusion proteins in axonal growth and transmitter release *Journal of Comparative Neurology* **367**: 222-234.

Pogson, D. M., D. L. Doxey, J. S. Gilmour, E. M. Milne and H. K. Chisholm (1992).Autonomic Neuron Degeneration in Equine Dysautonomia (Grass Sickness) *Journal of Comparative Pathology* **107**: 271-283.

Poxton, I. R. (1984).Demonstration of the common antigens of Clostridium botulinum, C. sporogenes and C. novyi by an enzyme-linked immunosorbent assay and electroblot transfer *J Gen Microbiol* **130**: 975-981.

Poxton, I. R. and M. D. Byrne (1984).Demonstration of Shared Antigens in the Genus Clostridium by an Enzyme-Linked Immunosorbent-Assay *Journal of Medical Microbiology* **17**: 171-176.

Poxton, I. R., L. C. Hunter, R. Brown, H. G. Lough and J. K. Miller (1997). Clostridia and Equine Grass Sickness *Reviews in Medical Microbiology* **8** supplement 1: S49-S51.

Rocke, T. E. (1993). *Clostridium botulinum. In* C. L. Gyles, and C. O. Thoen (ed.), Pathogenesis of Bacterial Infections in Animals. Iowa State University Press. 86-98.

Rossetto, O., G. Schiavo, C. Montecucco, B. Poulain, F. Deloye, L. Lozzi and C. C. Shone (1994).Snare Motif and Neurotoxins *Nature* **372**: 415-416.

Sakaguchi, Y., T. Hayashi, K. Kurokawa, K. Nakayama, K. Oshima, Y. Fujinaga, M. Ohnishi, E. Ohtsubo, M. Hattori and K. Oguma (2005).The genome sequence of Clostridium botulinum type C neurotoxin-converting phage and the molecular mechanisms of unstable lysogeny *Proceedings of the National Academy of Sciences of the United States of America* **102**: 17472-17477.

Schiavo, G. M. and C. Montecucco (1997). Clostridial Neurotoxins. In: Bacterial Toxins Tools in Cell Biology and Pharmacology, edited by K. Aktories p169-192. Chapman and Hall.

Schiavo, G., M. Matteoli and C. Montecucco (2000).Neurotoxins affecting neuroexocytosis *Physiological Reviews* **80**: 717-766.

Schiavo, G., O. Rossetto, S. Catsicas, P. P. Delaureto, B. R. Dasgupta, F. Benfenati and C. Montecucco (1993).Identification of the Nerve-Terminal Targets of Botulinum Neurotoxin Serotype-a, Serotype-D, and Serotype-E *Journal of Biological Chemistry* **268**: 23784-23787.

Schmid, A., R. Benz, I. Just and K. Aktories (1994).Interaction of Clostridium-Botulinum C2 Toxin with Lipid Bilayer-Membranes - Formation of Cation-Selective Channels and Inhibition of Channel Function by Chloroquine *Journal of Biological Chemistry* **269**: 16706-16711.

Scholes, S. F. E., C. Vaillant, P. Peacock, G. B. Edwards and D. F. Kelly (1993).Enteric Neuropathy in Horses with Grass Sickness *Veterinary Record* **132**: 647-651.

Sehr, P., G. Joseph, H. Genth, I. Just, E. Pick and K. Aktories (1998).Glucosylation and ADP ribosylation of Rho proteins: Effects on nucleotide binding, GTPase activity, and effector coupling *Biochemistry* **37**: 5296-5304.

Selzer, J., F. Hofmann, G. Rex, M. Wilm, M. Mann, I. Just and K. Aktories (1996).Clostridium novyi alpha-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins *Journal of Biological Chemistry* **271**: 25173-25177.

Shone, C. C., P. Hambleton and J. Melling (1985).Inactivation of Clostridium-Botulinum Type-a Neurotoxin by Trypsin and Purification of 2 Tryptic Fragments -

Proteolytic Action near the Cooh-Terminus of the Heavy Subunit Destroys Toxin-Binding Activity *European Journal of Biochemistry* **151**: 75-82.

Simpson, L. L. (1989). Peripheral Actions of the Botulinum Toxins. In: Botulinum Neurotoxin and Tetanus Toxin, p153-178, edited by L. L. Simpson. New York Academic Press.

Simpson, L. L. (1982).A Comparison of the Pharmacological Properties of Clostridium-Botulinum Type-C1 and Type-C2 Toxins *Journal of Pharmacology and Experimental Therapeutics* **223**: 695-701.

Smith, L. D. (1975). *Clostridium novyi*. In: The Pathogenic Anaerobic Bacteria, edited by A. Balows, 2nd edition, p257-270. CC Thomas: Springfield, Illinois.

Smith, G. R. and A. Turner (1989).The Production of Clostridium-Botulinum Toxin in Mammalian, Avian and Piscine Carrion *Epidemiology and Infection* **102**: 467-471.

Songer, J. G., and D. Bueschel. (1999). Multiplex PCR procedure for genotyping *Clostridium perfringens*. URL: http://www.microvet.arizona.edu/research/Clostridium_Web/multiplexprocedure.doc (accessed 1 March, 2005).

Stewart, W. J. (1977).Case of Suspected Acute Grass Sickness in a Thoroughbred Mare *Australian Veterinary Journal* **53**: 196-196.

Strom, M. S., M. W. Eklund and F. T. Poysky (1984).Plasmids in Clostridium-Botulinum and Related Clostridium Species *Applied and Environmental Microbiology* **48**: 956-963.

Sugiyama, H. (1980).Clostridium-Botulinum Neurotoxin *Microbiological Reviews* **44**: 419-448.

Sunagawa, H. and K. Inoue (1991).Isolation and Characterization of Converting and Non-Converting Phages, Harbored in the Strains of Clostridium-Botulinum Type-C and Type-D Isolated in Japan *Journal of Veterinary Medical Science* **53**: 951-954.

Sunagawa, H. and K. Inoue (1992).Biological and Biophysical Characteristics of Phages Isolated from Clostridium-Botulinum Type-C and Type-D Strains, and Physicochemical Properties of the Phage Dnas *Journal of Veterinary Medical Science* **54**: 675-684.

Sunagawa, H., T. Ohyama, T. Watanabe and K. Inoue (1992).The Complete Amino-Acid-Sequence of the Clostridium-Botulinum Type-D Neurotoxin, Deduced by Nucleotide-Sequence Analysis of the Encoding Phage D-16-Phi Genome *Journal of Veterinary Medical Science* **54**: 905-913.

Tocher, J. F., W. Tocher, W. Brown and J. B. Buxton (1923). Grass Sickness Investigation Report *Veterinary Record* **3**: 37-45 and 75-89.

Uzal, F. A. and C. A. Robles (1993).Mal Seco, a Grass Sickness-Like Syndrome of Horses in Argentina *Veterinary Research Communications* **17**: 449-457.

vonEichelStreiber, C., P. Boquet, M. Sauerborn and M. Thelestam (1996).Large clostridial cytotoxins - A family of glycosyltransferases modifying small GTP-binding proteins *Trends in Microbiology* **4**: 375-382.

Whitlock, R. H. and C. Buckley (1997).Botulism *Veterinary Clinics of North America-Equine Practice* **13**: 107-&.

Williamson, J. L., T. E. Rocke and J. M. Aiken (1999).In situ detection of the Clostridium botulinum type C-1 toxin gene in wetland sediments with a nested PCR assay *Applied and Environmental Microbiology* **65**: 3240-3243.

Williamson, L. C., J. Halpern, V. Dunlap and E. A. Neale (1995).Botulinum Neurotoxin-C Acts on Syntaxin and Snap-25 and Is Cytotoxic to Neurons *Molecular Biology of the Cell* **6**: 358-358.

Williamson, L. C. and E. A. Neale (1998).Syntaxin and 25-kDa synaptosomal-associated protein: Differential effects of botulinum neurotoxins C1 and A on neuronal survival *Journal of Neuroscience Research* **52**: 569-583.

Wood, J. L. N., E. M. Milne and D. L. Doxey (1998).A case-control study of grass sickness (equine dysautonomia) in the United Kingdom *Veterinary Journal* **156**: 7-14.

Woody, R. C. and E. M. Ross (1989).Neonatal Tetanus (St-Kilda, 19th-Century) *Lancet* **1**: 1339-1339.

Yamakawa, K., S. Nishida and S. Nakamura (1983).C2 Toxicity in Extract of Clostridium-Botulinum Type-C Spores *Infection and Immunity* **41**: 858-860.

Yokosawa, N., Y. Kurokawa, K. Tsuzuki, B. Syuto, N. Fujii, K. Kimura and K. Oguma (1989).Binding of Clostridium-Botulinum Type-C Neurotoxin to Different Neuro-Blastoma Cell-Lines *Infection and Immunity* **57**: 272-277.

Zhou, Y., H. Sugiyama and E. A. Johnson (1993).Transfer of Neurotoxigenicity from Clostridium-Butyricum to a Nontoxigenic Clostridium-Botulinum Type E-Like Strain *Applied and Environmental Microbiology* **59**: 3825-3831.

Journal of Medical Microbiology (2007), **56**, 196–201 DOI 10.1099/jmm.0.46802-0

A PCR approach to determine the distribution of toxin genes in closely related *Clostridium* species: *Clostridium botulinum* type C and D neurotoxins and C2 toxin, and *Clostridium novyi* α toxin

Ann Heffron and Ian R. Poxton

Correspondence
Ian R. Poxton
i.r.poxton@ed.ac.uk

Medical Microbiology, Centre for Infectious Diseases, University of Edinburgh College of Medicine and Veterinary Medicine, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK

The closely related *Clostridium novyi* and *Clostridium botulinum* types C and D are of current interest because of their association with serious infections in injecting drug users (*C. novyi* type A) and equine and feline dysautonomias (*C. botulinum* types C/D). The species are defined by the major toxins they produce: the α toxin of *C. novyi*, and the type C and D neurotoxins of *C. botulinum* (BoNT/C and BoNT/D). The other major toxin produced by this group, and previously thought to be restricted to the botulinum types, is the chromosomally encoded C2 – a binary toxin consisting of two components, I and II. In the current study 44 of these clostridia from the authors' culture collection were investigated – most of which had been identified previously by conventional biochemical tests as '*C. novyi* type A'. The aim was to check the distribution of toxin genes by PCR to see if the identities were consistent with the genes carried, and to ascertain if the C2 gene was only found in authentic *C. botulinum* strains. Several combinations of the species-defining genes and the two components of the C2 genes were detected. Only the authentic BoNT/C- and BoNT/D-positive *C. botulinum* strains and one of two non-neurotoxic variants of type C carried genes for both components of the C2 toxin. Of the remaining 40 *C. novyi* type A-like strains, the gene for the α toxin was found in 22, with 19 of these also possessing the gene for component I (16) or component II (3) but not both. In the α toxin-negative strains (22), both of the C2 genes were detected in 5 strains (3 *C. botulinum*), with component I in 11 strains and neither gene in 6 strains.

Received 27 June 2006
Accepted 19 October 2006

INTRODUCTION

Clostridium botulinum type C and D (group III *C. botulinum*) and the non-neurotoxigenic *Clostridium novyi* type A are phenotypically similar and cannot be distinguished by traditional methods of culture or biochemical properties – including GLC fatty acid profiles (Hunter & Poxton, 2002). Immunologically they share many cross-reactive surface antigens (Poxton & Byrne, 1984). Identification of this group of organisms to the species level is by the detection of the major toxin they produce: the botulinum neurotoxin C (BoNT/C) or D (BoNT/D) for *C. botulinum* and the α toxin for *C. novyi*. Traditionally this was done by observing their lethal effects in animal models – usually with specific neutralization by anti-toxin, and this is still the only standard method for definitive identification of *C. botulinum* types.

The genes encoding the major toxin of this group of species are carried on pseudolysogenic bacteriophages and the host–phage relationship is unstable. The phage is readily lost during subculture and sporulation, and it is thought that a cycle of phage loss and reinfection occurs *in vivo*. Interconversion of toxin types also occurs between type C and type D strains, and *C. novyi* type A. Furthermore, non-toxigenic strains can be converted to type C or D or *C. novyi* type A by infection with the converting phages. Infection with one phage confers immunity to the other phage suggesting that the two phages are closely related (Sunagawa & Inoue, 1991, 1992; Eklund *et al.*, 1974; Hunter & Poxton, 2002).

The group III *C. botulinum* members also produce a binary ADP-ribosylating cytotoxin, the C2 toxin. This consists of two separate components: the enzymic component, C2I, and the transport component, C2II (Ohishi *et al.* 1980). The genes for this toxin are located on the bacterial chromosome and are produced by the majority of type C and some type D strains (Fujii *et al.*, 1996; Kimura *et al.*, 1998). It is produced during sporulation and may form part of the spore coat (Nakamura *et al.*, 1978).

C. botulinum C and D are well known for causing botulism in animals, particularly in birds, but not in humans (Hatheway, 1995). *C. novyi* is recognized as a rare cause

Abbreviation: IDU, injecting drug user.

of wound and soft tissue infections in humans, classically war wounds, and in farm animals, but the species has been rarely encountered in clinical medicine over the past 50 years (Poxton, 2005). However, recently, awareness of these organisms has increased: *C. novyi* has been encountered in lethal infections in injecting drug users (IDUs) (McGuigan *et al.*, 2002), and *C. botulinum* C and possibly D has been identified as the probable aetiological agent of equine dysautonomia/equine grass sickness (Hunter *et al.*, 1999; McCarthy *et al.*, 2004) and of feline dysautonomia/Key-Gaskell syndrome (Nunn *et al.*, 2004) – both characterized by damage occurring to the autonomic nervous system – as revealed by histopathology.

In preliminary studies to develop PCR methods to detect the genes that encode the toxins one or both components of the C2 toxin genes were detected in some isolates previously identified as *C. novyi* type A. As this cytotoxin may have an important role in the pathogenesis of the conditions described above, it prompted us to examine our laboratory collection of *C. novyi* and related strains, previously identified by a range of phenotypic characteristics, for the presence of genes encoding the C2 toxin as well as the characteristic major toxins of the group: the neurotoxins BoNT/C and BoNT/D, and the *C. novyi* α toxin.

METHODS

Bacterial strains and culture. A total of 44 strains from our lyophilized culture collection were investigated in this study and are listed in Table 1. The strains included 40 labelled *C. novyi* type A or *C. novyi*-like, together with 1 BoNT/C-positive and 1 BoNT/D-positive *C. botulinum* strain and 2 BoNT/C-negative *C. botulinum* type C strains. These BoNT/C-negative stains had been considered neurotoxigenic when originally deposited in the National Collection of Type Cultures. It is unclear how all the strains had been identified as they were collected over 40 years. All had been identified by R. Brown utilizing biochemical reactions, including the detection of fatty acid end products by GC, since about 1977 and the identification keys used are described in Collee *et al.* (1996).

All strains were cultured in 5 ml anaerobic identification medium broth (Brown *et al.*, 1996) with cooked meat particles and incubated under anaerobic conditions (Mark III or MACS Anaerobic Workstations, Don Whitley Scientific) at 37 C for 24 h and subcultured onto Fastidious Anaerobe Agar (LabM) supplemented with 10 % egg yolk emulsion (Oxoid) for overnight incubation.

Template preparation. Ten large colonies were picked from each plate and emulsified in 100 µl 5 % suspension Chelex 100 (Bio-Rad) following the method of de Lamballerie *et al.* (1992). Samples were heated at 100 C for 10 min to release DNA and centrifuged at 1000 ***g*** for 5 min. A volume of 5 µl each supernatant was used as the template in the PCR mix.

DNA amplification. PCR was performed in a reaction volume of 50 µl containing 1 × REDTaq PCR buffer (Sigma), 1.5 mM $MgCl_2$ (3.75 mM for BoNT/C), 0.2 mM dNTP mixture, 2 U REDTaq DNA polymerase, 10 pmol each primer and 5 µl each DNA template. All primers were synthesized by MWG Biotech (http://www.mwg-biotech.com) and their sequences and reference source are detailed in Table 2. All amplification reactions were carried out in a PCR thermocycler (Techne TC-412). Cycling conditions are given in Table 3. The amplified products were separated by electrophoresis in 1 % agarose gels and stained with ethidium bromide. DNA ladder (ReadyLoad 100 bp, Invitrogen) was used as a molecular mass marker to indicate the sizes of the amplified products.

RESULTS AND DISCUSSION

The type strains of *C. botulinum* type C and D, 4564 (NCTC 373) and 3923 (NCTC 8265), respectively, were the only strains in which the genes for the C and D neurotoxins were detected (Fig. 1a). These also carried the genes for both components of the C2 toxin (Fig. 1b), but note the size of the PCR product for the type D strain was larger than for that found in the type C or *C. novyi* strains. However, sequencing and a BLAST search identified it as the gene for C2 component I (results not shown). Two strains originally supplied by NCTC as type C cultures 2510 (NCTC 10914) and 4565 (NCTC 3732) had previously been shown to be BoNT/C-negative by use of a specific ELISA for the toxin and this was confirmed by PCR. Both of the neurotoxin-producing strains also yielded the expected amplification products for both genes of the components of C2 toxin, as did one of the BoNT/C-negative strains. However, the other, 4565, yielded the C2 component I gene but not component II. The loss of the neurotoxins has been acknowledged by NCTC.

Of the 40 strains originally named as *C. novyi* type A or *C. novyi* type A-like, only 22 (55 %) tested positive for the *C. novyi* α toxin gene by PCR. All negative strains were tested on at least two further occasions with consistently negative results for the α toxin gene. Whether this result is real or due to sequence mismatch at the site of primers is not yet known. Further work involving sequence determination of several different α toxin genes and the design of new primers would clarify this point. Unexpectedly, however, 28 of the *C. novyi* strains (77 %) contained the gene for the C2CI toxin (these included 16 of the 22 α toxin-positive strains). Five '*C. novyi*' strains (12.5 %) contained the gene for the C2CII toxin (three α toxin-positive and two negative) and nine (22.5 %) were negative for both components. No α toxin-positive isolates contained both of the C2 components. The overall distribution of gene profiles is indicated in Table 1 and is summarized in Table 4.

As far as we can ascertain few, if any, of the so-called *C. novyi* strains had been tested in our laboratory specifically for the α toxin when originally identified, although some guinea pig inoculations had been performed in the 1970s but records are sketchy. However, all gave biochemical profiles – including volatile fatty acid patterns typical of *C. novyi* type A, but the gene encoding the α toxin was detected in only just over half of our collection.

It is noteworthy that all of the strains in our collection that had been isolated from cases of infection in IDUs, with characteristic pathology, were found to contain the gene for the α toxin. In 2000 there were 60 cases of extremely serious infection in Scotland with 23 deaths, with *C. novyi* definitely

Table 1. List of clostridial strains used and toxins identified

Lab no.	Original identity	Source	Toxin profile (C1 D C2I C2II α)*
141	*C. novyi* type A-like	Horse ileum, subacute EGS	− − + − −
465	*C. novyi* type A	Wound site, common bile duct drain	− − + − −
2306	*C. novyi* type A	?	− − + − −
2307	*C. novyi* type A	?	− − + + −
2510	*C. botulinum* type C†	NCTC 3732	− − + + −
2530	*C. novyi* type A	NCTC 538	− − + − +
2531	*C. novyi* type A	Glaxo collection	− − + − +
2533	*C. novyi* type A	NCTC 6735	− − + − −
2534	*C. novyi* type A	?	− − + + −
2535	*C. novyi* type A	?	− − + − −
2536	*C. novyi* type A	?	− − + − +
3209	*C. novyi* type A	PHLS 2680	− − + − +
3341	*C. novyi* type A	Soil (St Kilda)	− − − − −
3923	*C. botulinum* type D	NCTC 8265	− + + + −
4540	*C. novyi* type A	Injection site (Glasgow)	− − + − +
4541	*C. novyi* type A	Injection site (Glasgow)	− − + − +
4545	*C. novyi* type A	Crush injury wound swab	− − + − +
4547	*C. novyi* type A	Injection site (PHLS Cardiff)	− − + − +
4548	*C. novyi* type A	Injection site (PHLS Cardiff)	− − + − +
4549	*C. novyi* type A	Horse ileum, AGS	− − + − −
4550	*C. novyi* type A	Horse ileum, AGS	− − − − −
4551	*C. novyi* type A	Horse ileum, AGS	− − + − −
4552	*C. novyi* type A	Hare with dysautonomia, small intestine	− − − − +
4553	*C. novyi* type A	Hare with dysautonomia, small intestine	− − − − −
4554	*C. novyi* type A	Hare with dysautonomia, small intestine	− − + − −
4556	*C. novyi* type A	Horse faeces, CGS	− − − − −
4557	*C. novyi* type A	Horse faeces, recovered CGS	− − − − −
4558	*C. novyi* type A	Horse faeces, contact CGS	− − + − −
4559	*C. novyi* type A	Horse faeces, contact CGS	− − − − −
4561	*C. novyi* type A	Horse ileum, AGS	− − + − −
4562	*C. novyi* type A	Horse ileum, CGS	− − + − +
4563	*C. novyi* type A	Horse faeces, CGS	− − + − +
4564	*C. botulinum* type C	NCTC 8548	+ − + + −
4565	*C. botulinum* type C†	NCTC 10914	− − + − −
4566	*C. novyi* type A	Injection site (Glasgow)	− − + − +
4567	*C. novyi* type A	Injection site (Glasgow)	− − + − +
4568	*C. novyi* type A	NCTC 6738	− − + − +
4569	*C. novyi* type A	Injection site (Dublin)	− − + − +
4570	*C. novyi* type A	ARU collection	− − − − +
4571	*C. novyi* type A	NCTC 538	− − + − +
4572	*C. novyi* type A	Injection site (Bournemouth)	− − − − +
4573	*C. novyi* type A	Injection site (Glasgow)	− − − + +
4574	*C. novyi* type A	Injection site (Glasgow)	− − − + +
4575	*C. novyi* type A	Injection site (Stockton)	− − − + +

EGS, Equine grass sickness; AGS, acute form of EGS; CGS, chronic form of EGS; PHSL, public health service laboratory; ARU, Anaerobe Reference Unit at Cardiff; ?, source unknown.

*Toxin profile: C1, BoNT/C; D, BoNT/D; C2I, C2 component I; C2II, C2 component II; α, *C. novyi* α toxin; +, positive; −, negative.

†Non-neurotoxin producer.

implicated in 23 cases and probably implicated in 37 cases as the cause. Spores of clostridia can contaminate the materials used by IDUs and when introduced into tissue in an anaerobic environment the spores can germinate and produce exotoxins. All of the cases in Scotland involved IDUs injecting a solution of street heroin and citric acid into muscle tissue (McGuigan *et al.*, 2002). The few samples of heroin cultured from this outbreak were negative for *C.*

Table 2. Primer sequences used for the detection of *C. botulinum* types C and D, *C. novyi* α and C2 components

Gene	Primer	Sequence	Reference
Type C neurotoxin	Tox-384	5′-AAACCTCCTCGAGTTACAAGCCC-3′	Williamson *et al.* (1999)
	Tox-850	5′-GAAAATCTACCCTCTCCTACATCA-3′	
Type D neurotoxin	ToxD-F	5′-GTGATCCTGTTAATGACAATG-3′	Sunagawa *et al.* (1992)
	ToxD-R	5′-TCCTTGCAATGTAAGGGATGC-3′	
C. novyi α toxin	Nov-F	5′-GGTGCGATTCAAGAGGCCACA-3′	Hofmann *et al.* (1995)
	Nov-R	5′-CGCTCCTAGCAGTCCCGAAAT-3′	
C2 component I	C2CI-F	5′-AAGGAAGATAAAACAAAAAT-3′	Fujii *et al.* (1996)
	C2CI-R	5′-CCTAATGATACAAATGAAAA-3′	
C2 component II	C2CII-F	5′-GCAGAAGTTTCAGGTAGTTTACAAC-3′	Based on sequence
	C2CII-R	5′-CGCATTCTATAACGACCTTCTGGA-3′	Kimura *et al.* (1998)

novyi (Jones *et al.*, 2002) but the circumstantial evidence was compelling that *C. novyi* type A was the main if not only agent involved in the majority of cases.

The isolates obtained from environmental and animal sources do not appear to have the same distribution of toxin genes. Only 3/14 of these animal isolates contained the α toxin gene though all were phenotypically similar.

None of the isolates obtained from IDUs contained both components of the C2 toxin, but the majority (62 %) contain only the gene for the enzymic component (C2CI). It is possible that the C2CII transport component is not needed for transport into an already-damaged cell with the active C2CI component alone contributing to the pathology. The animal and environmental isolates show a similar pattern with 43 % containing the enzyme component of the C2 toxin and no isolates containing the gene for the binding component. None of the strains in our collection contained both of the C2 component genes together with the α toxin genes. In fact the only strains containing genes for both components of C2 were the two type strains of neurotoxin producing *C. botulinum* (C and D) together with one of the BoNT/C-negative strains, and two *C. novyi* strains in which the α toxin genes were not detected (strains 2307 and 2534). Unfortunately the origin of these strains was not recorded but they could well represent *C. botulinum* strains that had lost their toxin-encoding phages.

Of the six strains in which none of the toxin genes was detected, one was from soil, and the others were from the gastrointestinal tracts of a hare with dysautonomia and four horses (two with active dysautonomia).

It is evident that the complement of toxin genes that is carried by isolates of this group of clostridia is extremely variable. To cause classical botulism, the specific neurotoxins must be produced. Similarly, typical clinical *C. novyi*-mediated disease almost certainly requires the α toxin to be produced to induce the classical oedematous lesions. However, depending on the type of clinical situation, it is possible that the C2 toxin alone, or in combination with α

Table 3. Cycling conditions used for the detection of *C. botulinum* types C and D, *C. novyi* α and C2 components

Primer	Initial denaturation	No. of cycles	Cycling condition	Final extension
Tox-384/Tox850	5 min at 80 °C	30	1 min at 95 °C 1 min at 55 °C 1 min at 72 °C	5 min at 72 °C
ToxD-F/ToxD-R	10 min at 95 °C	25	1 min at 94 °C 1 min at 55 °C 1 min at 72 °C	3 min at 72 °C
Nov-F/Nov-R	3 min at 94 °C	30	1 min at 95 °C 1 min at 48 °C 1 min at 72 °C	5 min at 72 °C
C2CI-F/C2CI-R	3 min at 94 °C	35	45 s at 94 °C 2 min at 45 °C 1 min at 72 °C	5 min at 72 °C
C2CII-F/C2CII-R	3 min at 94 °C	40	45 s at 94 °C 1 min at 53 °C 3 min at 72 °C	5 min at 72 °C

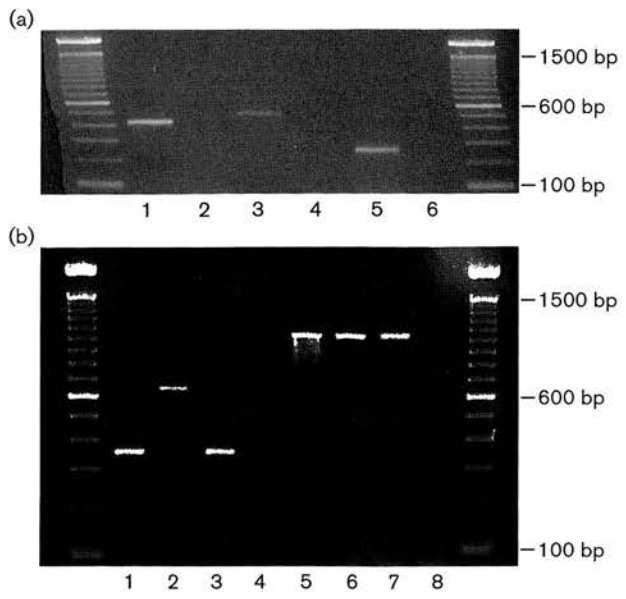

Fig. 1. (a) PCR for the genes encoding the two neurotoxins and *C. novyi* α toxin. Lanes 1 and 2, BoNT/C, strain 4564 and negative control; lanes 3 and 4, BoNT/D, strain 3923 and negative control; lanes 5 and 6, *C. novyi* α toxin, strain 2307 and negative control. Side lanes 100 bp DNA ladder. (b) PCR investigating the same three strains as in (a) for the genes encoding the two components of the C2 toxin. C2CI, lanes 1–4, strains 4564, 3923, 2307 and negative control; C2CII, lanes 5–8, strains 4564, 3923, 2307 and negative control. Note that the PCR product for the *C. botulinum* type D strain is larger than the *C. botulinum* type C and *C. novyi* type A strains.

toxin or neurotoxin, may contribute towards the pathology. If α toxin is present there does not seem to be a major requirement for the C2CII component. However, when in combination with the botulinum neurotoxin it seems that both components may be required.

As the various toxins – both neurotoxins and lethal toxins – are likely to affect the pathogenic potential of individual strains it is useful to know which ones they produce. However, this may be difficult because of the inherent instability of the pseudolysogenic bacteriophages that encode the two neurotoxins and the α toxin. In nature this does not matter as constant cycles of loss and reacquisition of the bacteriophages will presumably occur. Additionally, the process of sporulation may result in the bacteriophage being lost from the cell. It is therefore important to try to prevent loss during the initial isolation and early subculture of toxigenic strains. It is recommended that several colonies are selected and they are cultured in liquid medium to allow the infection–reinfection to take place and thereby prevent this.

In conclusion, this preliminary investigation by PCR – using previously published sequences for all but the C2 component

Table 4. Distribution of toxin genes in Group III clostridia

Profile of toxin genes	Number
C. novyi α^+, C2CI$^+$, C2CII$^+$	0
C. novyi α^+, C2CI$^+$, C2CII$^-$	16
C. novyi α^+, C2CI$^-$, C2CII$^-$	3
C. novyi α^+, C2CI$^-$, C2CII$^+$	3
C. novyi α^-, C2CI$^+$, C2CII$^+$	5*
C. novyi α^-, C2CI$^+$, C2CII$^-$	11†
C. novyi α^-, C2CI$^-$, C2CII$^-$	6

*Includes two *C. botulinum* type C (one BoNT/C+ and one BoNT/C−) and one *C. botulinum* type D (BoNT/D+).
†Includes one *C. botulinum* type C (BoNT/C−).

II gene – has shown the variety of combinations of toxin genes found in this group of related clostridia. The detection of at least one of the genes encoding the components of the C2 toxin in strains that appear to be *C. novyi* is novel and suggests a role for one or both of the components in the pathogenesis of these organisms. The failure to detect certain genes – especially the α toxin genes in strains previously identified as *C. novyi* – may be real, or could be due to polymorphisms in the genes for these toxins with the primers we have selected not matching adequately. This must be investigated in any future studies.

ACKNOWLEDGEMENTS

This work was supported by the Dubai Millennium Research Foundation. We are grateful to Fraser Pike and Robert Brown for additional assistance.

REFERENCES

Brown, R., Collee, J. G. & Poxton, I. R. (1996). *Bacteroides, Fusobacterium* and other Gram-negative anaerobic rods; anaerobic cocci; identification of anaerobes. In *Mackie and McCartney's Practical Medical Microbiology.* Edited by J. G. Collee, A. G. Fraser, B. P. Marmion & A. Simmonds. Edinburgh: Churchill Livingstone.

Collee, J. G., Brown, R. & Poxton, I. R. (1996). Clostridia of wound infection. In *Mackie and McCartney's Practical Medical Microbiology.* Edited by J. G. Collee, A. G. Fraser, B. P. Marmion & A. Simmonds. Edinburgh: Churchill Livingstone.

de Lamballerie, X., Zandotti, C., Vignoli, C., Bollet, C. & de Micco, P. (1992). A one-step microbial DNA extraction method using Chelex-100 suitable for gene amplification. *Res Microbiol* **143**, 785–790.

Eklund, M. W., Poysky, F. T., Meyers, J. A. & Pelroy, G. A. (1974). Interspecies conversion of *Clostridium botulinum* type-C to *Clostridium novyi* type-A by bacteriophage. *Science* **186**, 456–458.

Fujii, N., Kubota, T., Shirakawa, S., Kimura, K., Ohishi, I., Moriishi, K., Isogai, E. & Isogai, H. (1996). Characterization of component-I gene of botulinum C2 toxin and PCR detection of its gene in clostridial species. *Biochem Biophys Res Commun* **220**, 353–359.

Hatheway, C. L. (1995). Botulism: the present status of the disease. *Curr Top Microbiol Immunol* **195**, 55–75.

Hofmann, F., Herrmann, A., Habermann, E., and Von Eichel Streiber, C. (1995). Sequencing and analysis of the gene encoding the α-toxin of *Clostridium novyi* proves its homology to toxins A and B of *Clostridium difficile*. *Mol Gen Genet* **247**, 670–679.

Hunter, L. C. & Poxton, I. R. (2002). *Clostridium botulinum* types C and D and the closely related *Clostridium novyi*. *Rev Med Microbiol* **13**, 75–90.

Hunter, L. C., Miller, J. K. & Poxton, I. R. (1999). The association of *Clostridium botulinum* type C with equine grass sickness: a toxicoinfection? *Equine Vet J* **31**, 492–499.

Jones, J. A., Salmon, J. E., Djuretic, T., Nichols, G., George, R. C. & Gill, O. N. (2002). An outbreak of serious illness and death among injecting drug users in England during 2000. *J Med Microbiol* **51**, 978–984.

Kimura, K., Kubota, T., Ohishi, I., Isogai, E., Isogai, H. & Fujii, N. (1998). The gene for component-II of botulinum C2 toxin. *Vet Microbiol* **62**, 27–34.

McCarthy, H. E., French, N. P., Edwards, G. B., Poxton, I. R., Kelly, D. F., Payne-Johnson, C. E., Miller, K. & Proudman, C. J. (2004). Equine grass sickness is associated with low antibody levels to *Clostridium botulinum*: a matched case-control study. *Equine Vet J* **36**, 123–9.

McGuigan, C. C., Penrice, G. M., Gruer, L., Ahmed, S., Goldberg, D., Black, M., Salmon, J. E. & Hood, J. (2002). Lethal outbreak of infection with *Clostridium novyi* type A and other spore-forming organisms in Scottish injecting drug users. *J Med Microbiol* **51**, 971–977.

Nakamura, S., Serikawa, T., Yamakawa, K., Nishida, S., Kozaki, S. & Sakaguchi, G. (1978). Sporulation and C2 toxin production by *Clostridium botulinum* type-C strains producing no C1 toxin. *Microbiol Immunol* **22**, 591–596.

Nunn, F., Cave, T. A., Knottenbelt, C. & Poxton, I. R. (2004). Evidence that Key-Gaskell Syndrome is a toxico-infection caused by *Clostridium botulinum* type C/D. *Vet Rec* **155**, 111–115.

Ohishi, I., Iwasaki, M. & Sakaguchi, G. (1980). Purification and characterization of two components of botulinum C2 toxin. *Infect Immun* **30**, 668–673.

Poxton, I. R. (2005). Other *Clostridium* species. In *Principles and Practice of Clinical Bacteriology*, 2nd edn. Edited by S. H. Gillespie & P. M. Hawkey. Chichester: Wiley.

Poxton, I. R. & Byrne, M. D. (1984). Demonstration of shared antigens in the genus *Clostridium* by an enzyme-linked immunosorbent assay. *J Med Microbiol* **17**, 171–176.

Sunagawa, H. & Inoue, K. (1991). Isolation and characterization of converting and non-converting phages, harbored in the strains of *Clostridium botulinum* type C and type-D isolated in Japan. *J Vet Med Sci* **53**, 951–954.

Sunagawa, H. & Inoue, K. (1992). Biological and biophysical characteristics of phages isolated from *Clostridium botulinum* type-C and type-D strains, and physicochemical properties of the phage DNAs. *J Vet Med Sci* **54**, 675–684.

Sunagawa, H., Ohyama, T., Watanabe, T. & Inoue, K. (1992). The complete amino-acid-sequence of the *Clostridium botulinum* type-D neurotoxin, deduced by nucleotide-sequence analysis of the encoding phage D-16-phi genome. *J Vet Med Sci* **54**, 905–913.

Williamson, J. L., Rocke, T. E. & Aiken, J. M. (1999). *In situ* detection of the *Clostridium botulinum* type C-1 toxin gene in wetland sediments with a nested PCR assay. *Appl Environ Microbiol* **65**, 3240–3243.